THE EUKARYOTIC SMC5/6 COMPLEX REPRESSES THE REPLICATIVE PROGRAM OF HIGH-RISK HUMAN PAPILLOMAVIRUS

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DEDICATION

The great journey of my life will always have been my children, Ryan Alexandris and Atlis Kenneth Gibson. My sons, without you, your love and your smiles, I would not be the man I am today. I dedicate this work to you, my sons, my loves, my everythings.

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To my grandmother, Anne LaRue, and my Aunt, Lynn Witty, I will forever be grateful to you for your tireless guidance, support and unconditional love during this journey. I could not have done this without you and will always hold a special place in this journey for you.

Ryan Taylor Gibson

THE EUKARYOTIC SMC5/6 COMPLEX REPRESSES THE REPLICATIVE PROGRAM OF HIGH-RISK HUMAN PAPILLOMAVIRUS

Human papillomaviruses (HPVs) are non-enveloped, circular double-stranded DNA viruses that infect basal keratinocytes of stratified squamous epithelia. High-risk HPV (HR-HPV) infection causes nearly all cervical cancers and an increasing number of head and neck cancers. While prophylactic vaccinations have reduced the incidence of HPV infection and attributable cancers, currently there is no cure for pre-existing HPV infection. As such, HPV remains a global health threat and a better understanding of HPV biology remains of significant medical importance for identification of novel therapeutic targets.

The multi-subunit structural maintenance of chromosomes 5/6 complex (SMC5/6) is comprised of SMC5, SMC6 and NSE1-4. SMC5/6 is essential for homologous recombination DNA repair and reportedly functions as an antiviral factor during hepatitis B and herpes simplex-1 viral infections. Intriguingly, SMC5/6 has been found to associate with HR-HPV E2 proteins, which are multifunctional transcription factors essential to regulation of viral replication and transcription. The function of SMC5/6 associations with E2, as well as its role during HR-HPV infection remain unclear and we explored this question in the context of HR-HPV-31. SMC6 interacted with HPV-31 E2 and co-immunoprecipitation of SMC6/E2

complexes required the E2 transactivation domain, inferring SMC6 association is limited to the full-length E2 isoform. Depletion of SMC6 and NSE3 increased HPV replication and transcription in keratinocytes stably maintaining episomal HPV-31, suggesting that the SMC5/6 complex represses these processes. Neither SMC6 nor NSE3 co-IP the viral E1 DNA helicase alone or E1/E2 complexes but the association of SMC6 with E2 was reduced in the presence of E1, indicating that SMC6 competes with E1 for E2 binding. This infers that SMC6 repression of the viral replicative program may involve inhibiting initiation of viral replication by disrupting E2 interactions with E1. Chromatin immunoprecipitation determined that SMC6 is present on episomal HPV-31 genomes, alluding to a possible role for SMC5/6 in modifying the chromatin state of viral DNA. Taken together, these findings describe a novel function for SMC5/6 as a repressor of the HPV-31 replicative program.

Elliot Androphy, MD (Chair)

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ATP	Adenosine triphosphate
BPV	Bovine papillomavirus
cccDNA	covalently closed circular DNA
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
DBD	DNA binding domain
E1BS	E1 binding site
E2BS	E2 binding site
E1	HPV E1 DNA helicase
E2	HPV E2 transcription factor
E4	HPV E4 protein
E5	HPV E5 oncoprotein
E6	HPV E6 oncoprotein
E7	HPV E7 oncoprotein
HPV	Human papillomavirus
HR-DDR	Homologous recombination DNA damage response
HR-HPV	High-risk human papillomavirus
IP	Immunoprecipitation
KITE	kleisin interacting tandem winged-helix element
LCR	Long control region
NLS	Nuclear localization sequence

NSE	Non-structural of maintenance chromosome element
ORF	Open reading frame
Ori	Origin of replication
pE	Early promoter
pL	Late Promoter
poly(A)E	Late polyadenylation sequence
poly(A)L	Late polyadenylation sequence
SMC	Structural maintenance of chromosomes
SMI	Suspension-mediated infection
TAD	Transactivation domain
qPCR	Quantitative polymerase chain reaction

CHAPTER 1

INTRODUCTION

1.1 Papillomaviruses

Papillomaviruses (PVs) are non-enveloped, double-stranded circular DNA viruses belonging to the viral *Papillomaviridae* family. The *Papillomaviridae* family of viruses are amongst the most ancient of viral families, believed to have evolved approximately 450 million years ago [1]. PVs infect basal keratinocytes of the skin and mucosal epithelium and are transmitted horizontally when microabrasions compromise the integrity of the epithelial layers, enabling PV access to basal keratinocytes [2]. Human papillomaviruses (HPVs) represent the most extensively studied of PVs, however, 53 different genera of PVs have been identified and shown to infect multiple different animals with a species-restricted tropism [3]. Indeed, a variety of primates, birds, reptiles, and most recently fish have all been found to be infected by PVs [4].

1.1.1 Human Papillomaviruses

While over 53 different genera of PVs have been identified, only 5 genera (alpha, beta, gamma, mu, nu) are known to infect humans [5]. To date, roughly 200 different types of HPVs have been discovered and are classified within these genera based on nucleotide sequence similarity of the viral L1 gene [6]. Of the

five genera shown to infect humans, certain genera are considered to be “low-risk” (LR-HPVs) and others “high-risk” (HR-HPVs) based on their oncogenic potential (Table 1.1). LR-HPVs typically infect the cutaneous epithelia and demonstrate minimal carcinogenic potential, and as such LR-HPV infections usually only result in benign lesions (papillomas). HPVs belonging to the beta, gamma, mu, and nu genera are generally considered by the International Agency for Research on Cancer (IARC) to be low-risk, however, data increasingly suggests that beta PVs may play a role as co-carcinogens in the development on non-melanoma skin cancer [7]. Certain alpha HPVs (HPV-6, -11) also fall within the low-risk categorization, as infection with these mucosotropic HPVs can result in benign genital lesions but fail to progress towards malignancy. Twelve mucosotropic HPVs belonging to the alpha genera are classified as carcinogenic and referred to as high-risk HPVs (HR-HPVs), as these HR-HPVs have been shown to be etiological agents in multiple different cancers, such as cervical, anal, penile, and oropharyngeal cancers [8]. Of the HR-HPVs, HPV-16 and HPV-18 are causative in over 70% of HPV-related cancers [9].

Table 1.1. HPV genotypes and genera classified based on oncogenic risk.

HPV Type	Genera	Tropism	Oncogenic Risk
HPV-3	beta	cutaneous epithelia	low
HPV-5	beta	cutaneous epithelia	low
HPV-6	alpha	mucosal epithelia	low
HPV-8	beta	cutaneous epithelia	low
HPV-11	alpha	mucosal epithelia	low
HPV-16	alpha	mucosal epithelia	high
HPV-18	alpha	mucosal epithelia	high
HPV-31	alpha	mucosal epithelia	high
HPV-33	alpha	mucosal epithelia	high
HPV-35	alpha	mucosal epithelia	high
HPV-39	alpha	mucosal epithelia	high
HPV-45	alpha	mucosal epithelia	high
HPV-51	alpha	mucosal epithelia	high
HPV-52	alpha	mucosal epithelia	high
HPV-56	alpha	mucosal epithelia	high
HPV-58	alpha	mucosal epithelia	high
HPV-59	alpha	mucosal epithelia	high

1.1.2 Epidemiology

HPVs are the most common sexually transmitted human pathogens [10]. Infection with either LR- or HR-HPVs belonging to the alpha genera is believed to primarily occur through horizontal transmission via sexual exposure of a susceptible individual with an HPV-positive partner. As such, roughly 90% of individuals are estimated to become infected with a sexually transmitted HPV during their lifetime [11]. Globally, HPV prevalence is highest early in life at roughly 25 years of age and decreases as age increases [12]. The majority of infected individuals remain asymptomatic and clear the viral infection within 1 to 2 years [12, 13]. However, in those individuals that fail to clear infections, chronic HR-HPV infection can progress towards malignancy. While less frequent, vertical transmission of HPV is known to occur, however the extent of the long-term risks associated with mother-to-infant transmission remain unclear [14-16].

Despite the availability of highly effective prophylactic HPV vaccinations the need to vaccinate early in life, low uptake, public mistrust, as well as preclusive barriers in the global implementation of vaccination programs have allowed for HPV to persist as a significant medical pathogen. Epidemiological data support that HR-HPV remains a leading cause of cancers worldwide, causing roughly 5% of all cancers globally (nearly 630,000 cases annually) and accounting for nearly 30% of cancer cases attributed to infectious agents in both men and women [9, 17]. The majority of HPV-related cancers are diagnosed in women and the

highest global burden occurs in less developed, lower-income regions of the world [9, 18] (Figure 1.1). Currently, no curative treatment exists for those with chronic HPV infection.

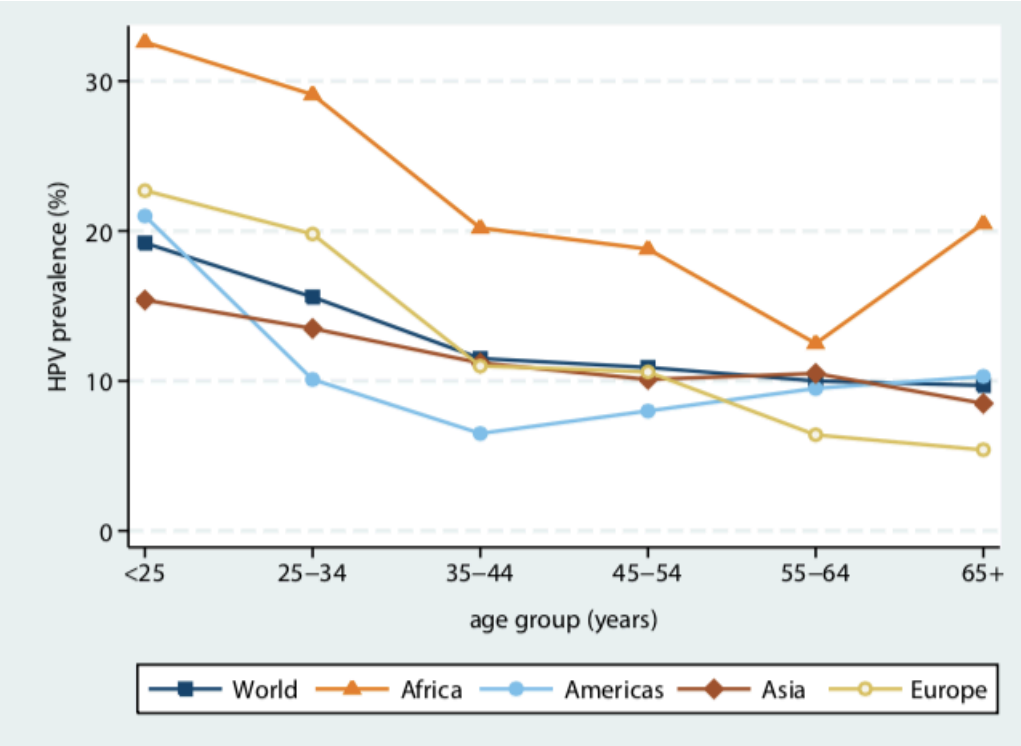

Figure 1.1. Global HPV prevalence. Rough age-specific global and regional prevalence of HPV infection in women. This figure was taken from [12].

1.1.3 Prevention

The most effectual means of preventing HPV-related disease is by blocking initial infection through the use of prophylactic vaccines. Currently, 3 vaccines are available and all are made using recombinant viral-like particles (VLPs) comprised of the HPV major capsid protein, L1 [19]. L1 has been shown to spontaneously self-assemble into icosahedral capsids that are identical in size and structure to the native HPV virion [20-24] and are highly immunogenic [21](Figure 1.2). As such, each VLP-based vaccine has demonstrated greater than 90% efficacy in preventing infection by the HPV types for which they are indicated [25-27] (Table 1.2). While highly effective at preventing infection there is no therapeutic benefit in administering the vaccine to previously infected individuals [28-30]. As such, the WHO and CDC recommend vaccination of both girls and boys early in life, as well as later for individuals not previously vaccinated (CDC, 2016).

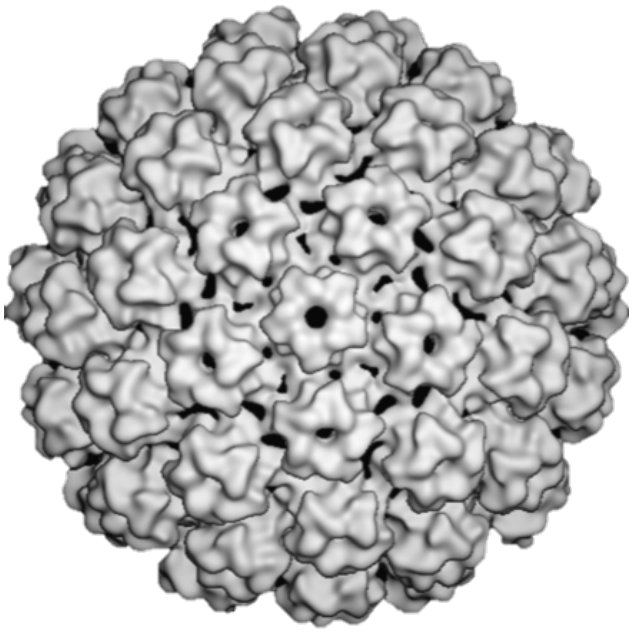

Figure 1.2. HPV-16 L1 VLP reconstructed by cryo-EM. HPV-16 L1 viral-like particle (VLP) reconstructed by cryo-EM when calculated at 18 Å. This image was taken from [31].

Table 1.2. FDA-licensed HPV vaccines.

VACCINE	HPV L1 TYPE	PRODUCER CELL	FDA LICENSURE
Cervarix	-16, -18	*Trichoplusia ni (Hi 5)* insect cell	2009
Gardasil	-6, -11, -16, -18	*Saccharomyces cerevesiae* yeast cell	2006
Gardasil 9	-6, -11, 16, -18, -31, -33, -45, -52, -58	*Saccharomyces cerevesiae* yeast cell	2014

1.2 High-Risk HPV Genome

HPVs possess a closed, circular double-stranded DNA genome roughly 8 kilobases in size that is maintained as an episome in the nuclei of infected keratinocytes [32] (Figure 1.3). The viral genome is comprised of three primary regions: the long control region (LCR), early region, and late region [7]. The LCR contains the viral origin of replication (ori), enhancer and silencer sequences, early promoter, HPV E1 and E2 protein binding sites, as well as sites necessary for the binding of various cellular factors involved in regulating replication and transcription of the viral genome [33]. A late promoter located downstream of the LCR in the E7 ORF is only activated upon keratinocyte differentiation in the later stages of the viral life cycle [34]. The early region contains 6 of the 8 HPV open reading frames (ORFs) which are transcribed from the early promoter located upstream of the viral E6 gene. These ORFs encode for the 6 viral proteins designated as early (E) (E1, E2, E4, E5, E6, E7). With the exception of E4, the descriptor of E was assigned due to their expression throughout the viral life cycle. E4 was termed an early protein due to the location of the E4 ORF being within the early region of the viral genome amongst the true viral early proteins [35]. However, E4 is only detected in the later stages of the viral life cycle and no early functions have been confirmed. The true early proteins (E1, E2, E5, E6, E7) execute multiple functions during the viral life cycle, such as regulating transcription and replication, immune evasion and maintaining infected cells in a proliferative state [36]. The late region encodes the HPV late (L) proteins (L1, L2)

that encapsidate viral genomes in the outer layers of the epithelium prior to shedding of infectious HPV virions.

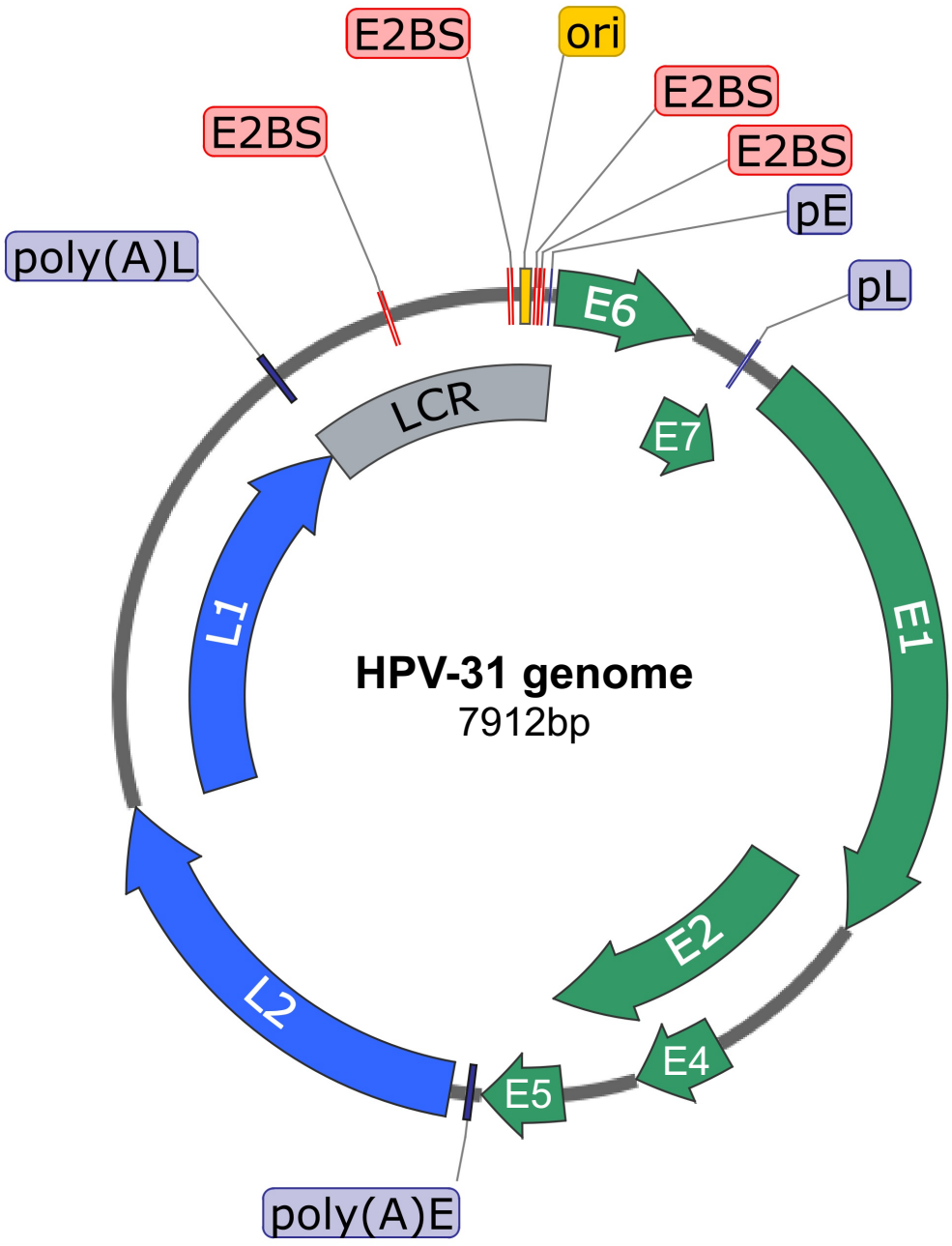

Figure 1.3. HPV-31 genome. The HPV-31 genome is roughly 8 kb in length and encodes for 8 viral proteins transcribed from viral early (pE) and late (pL) promoters and terminated at early (poly(A)E) or late (poly(A)L) polyadenylation sequences. The viral LCR contains the keratinocyte-specific enhancer, putative E2 and E1 binding sites, viral ori and early promoter.

1.2.1 Viral Proteins

The 8 proteins encoded by HPV perform multiple functions that enable completion of the viral life cycle. Protein expression is tightly regulated based on the phase of the viral replicative program and state of keratinocyte differentiation. Aside from categorization as early or late, the viral proteins can be further segregated by function; the replicative proteins (E1, E2), oncogenic proteins (E5, E6, E7), structural (capsid) proteins (L1, L2), and the less well understood E4 protein, which is believed to play a role in genome amplification [37-40] and potentially in the production of infectious virions [35]. While the E1 DNA helicase and E2 transcription factor are sufficient for transient viral replication [41-43], stable replication also requires E6 and E7 oncoproteins [44, 45]. The temporal control of viral protein expression prevents over-amplification of viral genomes and the expression of the highly immunogenic L1 capsid protein that could otherwise trigger an immune response.

1.2.2 HPV E1

The viral E1 ATP-dependent DNA helicase is the most highly conserved protein among PVs and only encoded protein to possess enzymatic activity, belonging to the superfamily III (SF3) family of AAA+ (ATPases Associated with various cellular Activities) helicases [46]. E1 is essential for PV replication throughout the viral life cycle, being required for the initial replication and establishment of viral infection, replicative maintenance, and amplification of viral genomes. Additionally, E1 may also play a role in HPV infection by modulating host-gene expression to inhibit the host anti-viral response [47].

Structurally, E1 is comprised of 3 functional regions, an N-terminal regulatory region (NRR), DNA-binding domain and C-terminal enzymatic domain [46] (Figure 1.4). The NRR is essential for E1-mediated viral replication *in vivo* and contains the E1 nuclear localization (NLS) and export (NES) signals. Phosphorylation of the NLS and NES regulate E1 nucleocytoplasmic shuttling [48-54]. The DNA-binding domain of E1 functions to recognize the viral ori and E1 binding sites adjacent the viral ori but has been shown to demonstrate poor specificity [46]. The C-terminal domain of E1 contains the oligomerization domain, Walker A/B ATPase domain needed for catalyzing ATP hydrolysis and the unwinding of DNA, and C-terminal brace. The C-terminal brace functions in the stabilization and assembly of E1 hexamers [55]. Both the DNA-binding

domain and C-terminal enzymatic domains have been shown to be essential for HPV replication *in vitro* [56].

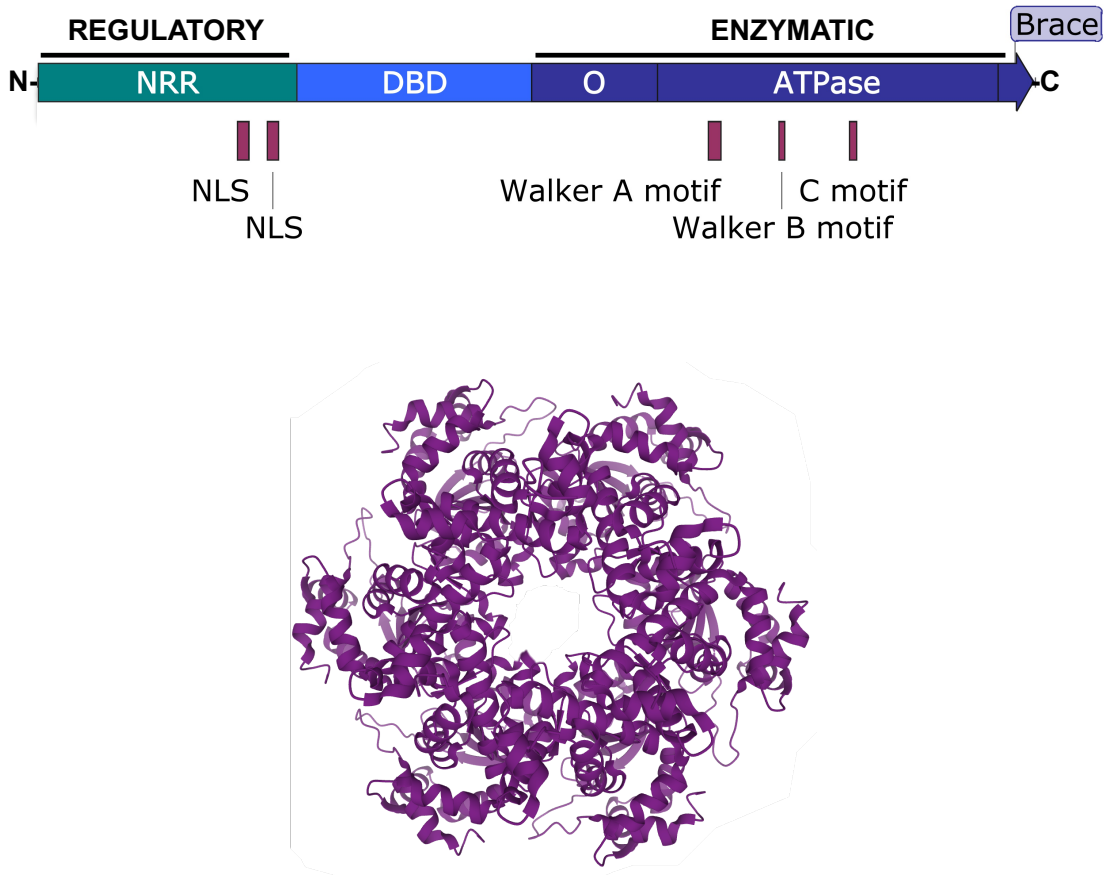

Figure 1.4. HPV E1. (**Top**) The N-terminal regulatory region of E1 (NRR- green) contains the viral nuclear localization signals (NLS). The DNA-binding domain (blue) recognizes E1 binding sites in the viral genome LCR region. The enzymatic region (purple) of E1 consists of an oligomerization domain (O), ATPase domain and C-terminal brace. (**Bottom**) Crystal structure of the PV E1 helicase domain hexamer at 3.15 Å (PDB ID: 2GXA, [57], Mol*, RCSB PDB).

E1 initiates viral replication through both its helicase activity and interactions with cellular replication factors [46] (Figure 1.5). The initiation of bi-directional viral DNA replication requires recruitment of E1 to the viral ori by E2, as the poor specificity for E1 DNA-binding precludes efficient viral replication in the absence of the enhanced specificity provided by E2 [58-62]. E2-mediates E1 recruitment to the viral ori by binding the C-terminal helicase domain (HD) of E1 using the E2 TAD [63-67], then directing it to the viral ori via the E2 DNA-binding domain [46]. E2 binding to the E1 HD also serves to inhibit non-specific binding of E1 with cellular DNA [68, 69]. Following E2-mediated recruitment of E1 to the viral LCR, the E1 DBD binds E1BS adjacent to the viral ori, for which a transient E1/E2-DNA ternary complex is formed prior to the release of E2 from the complex and viral genome [46, 70-73]. E1 then forms a double-trimer and hydrolyzes ATP, which opens the viral ori, displaces E2, and allows for the formation of the E1 double hexamer [71, 73-76]. E1 hexamers then proceed to bi-directionally unwind viral DNA using ATP hydrolysis. During this process E1 also recruits host factors needed for replicating viral genomes, such as DNA polymerase α-primase [77] and RPA [78].

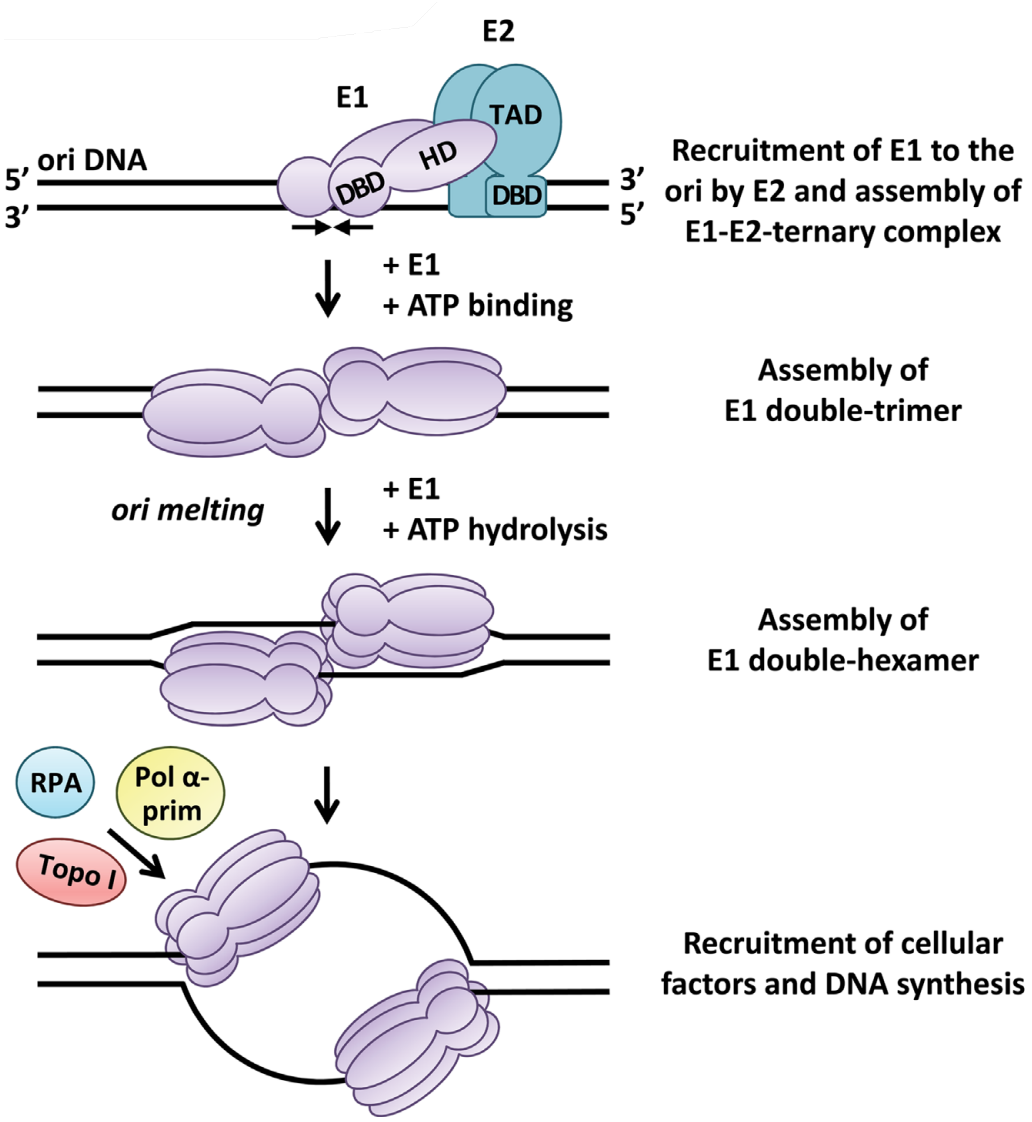

Figure 1.5. HPV replication initiation. HPV E1 is recruited to the viral ori and then form a double-trimer. Initial unwinding of the viral ori via ATP-hydrolysis causes displacement of E2 and the assembly of the E1 double-hexamer. Double-hexamers proceed to unwind DNA bi-directionally and E1 mediates recruitment of cellular replication factors to replicate viral DNA. This figure was taken from [46].

1.2.3 HPV E2

E2 is an essential multifunctional 40 kDa transcription factor that is expressed throughout the viral life cycle [79]. It is the primary factor involved in regulating viral replication and transcription [59, 69, 80]. E2 is also required for the maintenance and equal partitioning of viral genomes to daughter cells following cell division [81-85]. Lacking intrinsic enzymatic activity E2 is reliant upon interactions with viral proteins and a multitude of host factors (Table A.1) to execute its functions during the viral life cycle [79].

Structurally, monomeric full-length E2 is comprised of an N-terminal transactivation domain (TAD) that is connected to its C-terminal DNA-binding domain (DBD) by a disordered hinge region [86-91] (Figure 1.6). Both the TAD and DBD of E2 are relatively well conserved across different PVs, however the hinge region is not conserved [79]. All HPVs possess the ability to produce an additional E2 isoform, designated E8^E2. E8^E2 retains both the hinge and DBD of full-length E2 but lacks the N-terminal TAD. Instead, E8^E2 is produced from a spliced transcript that links the sequence of an alternative reading frame within E1 to the E2 hinge. This results in a short (10-13 residue) N-terminal peptide (E8) being linked to the E2 hinge in place of the TAD. PV E2 proteins exist as highly stable homodimers (E2/E2, E8^E2/E8^E2) or heterodimers (E2/E8^E2) that are formed through the E2 DBD [86, 92-94] and following dimerization no further mixing of E2 monomers occurs [86]. E2/E8^E2 heterodimers are capable of

initiating viral DNA replication, as well as regulating gene transcription but unlike full-length E2 homodimers are defective in genome partitioning [79, 95-97].

E2 is essential for effectual initiation of viral replication [79] and is the main determinant for specificity during recruitment of replicative factors to the HPV LCR [98]. E2-mediated recruitment involves protein-protein interactions and the binding of E2 with specific DNA consensus motifs ($ACCGN_4CGGT$, $ACCN_6GGT$) located within the LCR [99-101]. As previously discussed, E2 initiates viral replication primarily by recruiting E1 to the viral ori [67, 102]. E2 recruitment of E1 to the ori enables efficient initiation of viral genome replication, as the poor sequence specificity of HPV E1 for E1BS within the viral LCR precludes efficient viral replication in the absence of E2. While artificially high levels of E1 can initiate viral genome replication *in vitro*, *in vivo* replication requires E2 to localize E1 to the ori [103]. E2 can also stimulate initiation of replication by displacing nucleosomes from the viral ori [104].

E2 is essential to the regulation of viral gene transcription and this is achieved by localizing host factors that activate or repress transcription to the viral LCR [79]. For example, E2 can stimulate transcription through recruitment of Brd4 [105] and SMARCA2 [106] to viral episomes. Alternatively, E2 can regulate viral transcription by sterically hindering the association of certain host factors with viral DNA, such as TATA-binding protein [107] and SP1 [108].

HPV transcription is also regulated by E8^E2-mediated repression [79]. E8^E2 represses transcription by competing with E2 for E2BS within the viral LCR, thereby preventing E2 from recruiting factors that would activate transcription [109-111]. Additionally, E8^E2 represses transcription through interactions with the corepressor complex, NCoR/SMRT [112, 113].

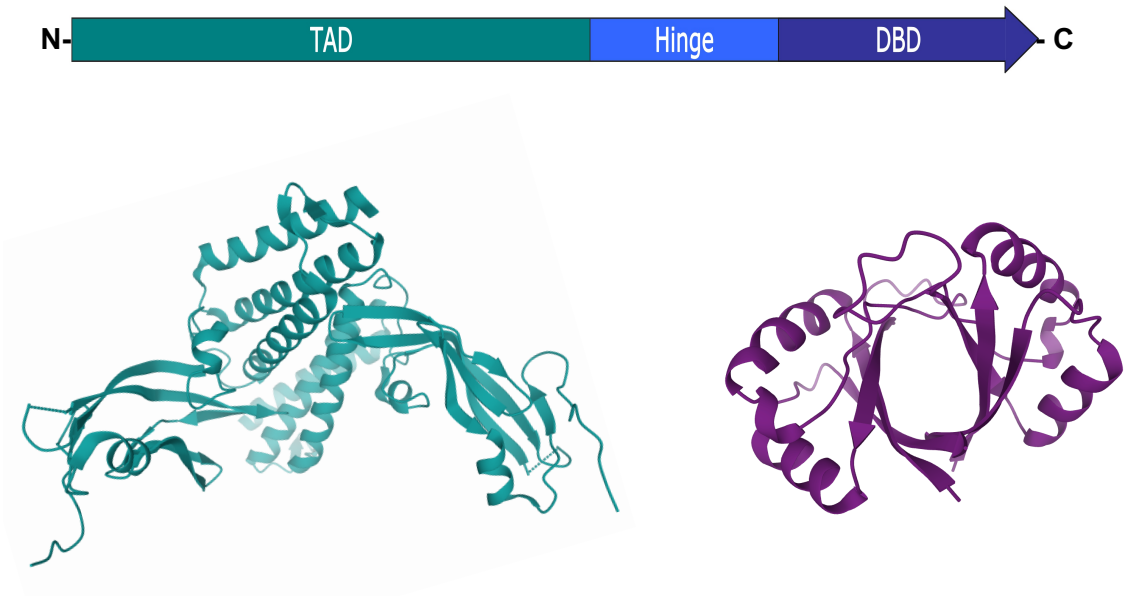

Figure 1.6. HPV E2. (**Top**) Full-length HPV E2 is comprised of an N-terminal transactivation domain (TAD- green) connected by a disordered linker (Hinge- blue) region to a C-terminal DNA binding/oligomerization domain (DBD- purple). (**Bottom left**) HPV-16 TAD crystal structure at 1.9 Å (PDB ID: 1DTO, [114], Mol*, RCSB PDB). (**Bottom right**) HPV-31 DBD crystal structure at 2.4 Å (PDB ID: 1A7G, [115], Mol*, RCSB PDB).

E2-mediated transcriptional regulation is also important in preventing cells from progressing towards malignancy. E2 regulates viral oncoprotein expression by repressing the early promoter and loss of E2 function results in overexpression of E6 and E7 [116-120]. While low levels of E6 and E7 are necessary for maintaining viral episomes and stable replication [45], overexpression of E6 and E7 promotes carcinogenesis [121].

1.3 HPV Life Cycle

HPV infection occurs when microabrasions compromise the integrity of the epithelium, enabling HPV virion access to basal keratinocytes (discussed in detail in Chapter 5.1)[122]. Following initial interactions of the HPV capsid with the cellular extracellular matrix, the viral L1 major capsid protein is cleaved by the KLK8 protease and then binds with the primary HPV receptor, heparin sulfate proteoglycans (HSPGs) (Figure 1.7). This binding induces conformational changes in the viral capsid that expose the L2 minor capsid protein to furin proteolytic cleavage. Cleavage of L2 induces conformational changes in the HPV capsid which are believed to increase the affinity of L1 for an unidentified cell surface receptor that mediates endocytic entry of HPV into host cells. Upon HPV entry into basal keratinocytes the viral capsid uncoats as endosomes mature and acidification increases. Furin-cleaved L2 then facilitates retrograde trafficking of viral genomes towards the nucleus in a process mediated by the host cell retromer complex [123]. Viral genomes reside within trafficking vesicles in the perinuclear space until breakdown of the nuclear envelope during mitosis [124-126]. Following nuclear envelope breakdown HPV DNA enters the cell nucleus and is localized to PML nuclear bodies by L2 [127-129].

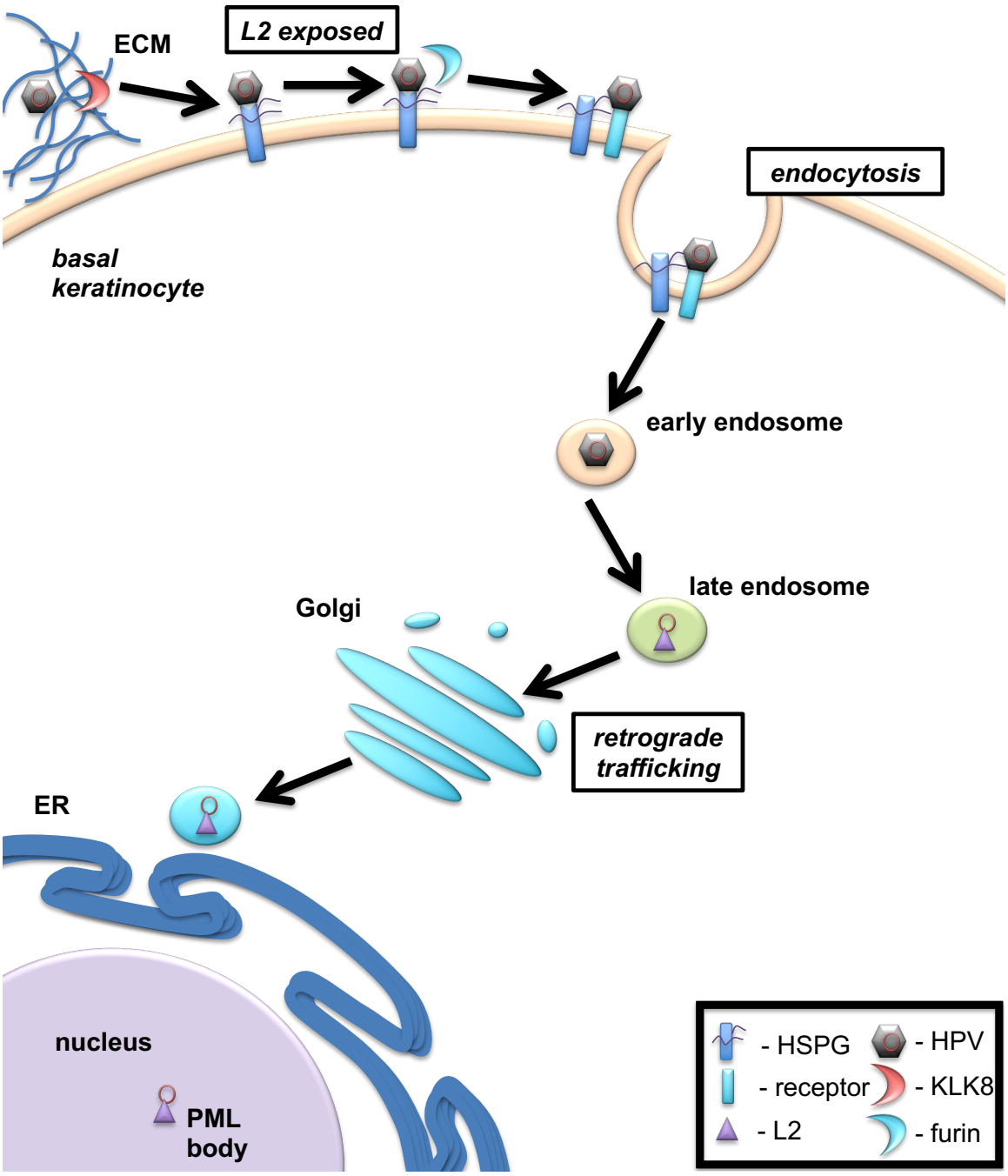

Figure 1.7. HPV infection and trafficking. HPV capsids are cleaved in the host cell ECM by the KLK8 protease. Capsids then bind heparin sulfate proteoglycans (HSPG) on the cell surface followed by cleavage of the L2 capsid protein. Internalization of viral nucleocapsids occurs after interactions with a secondary cellular receptor. Viral genomes are then trafficked from early to late endosomes where capsids will disassemble and L2 will mediate retrograde trafficking of viral genomes towards the nucleus. Upon breakdown of the nuclear envelope HPV genomes will be trafficked to PML nuclear bodies within the host cell nucleus.

HPV replication can be divided into 3 hypothetical phases; establishment, maintenance, and amplification [130] (Figure 1.8). The phase of the viral replicative program is tied to the level of host epithelial cell differentiation, transitioning from one to the next as the infected host cell progresses through the epithelial layers towards terminal differentiation. However, precisely how the virus is stimulated to switch from one phase of replication to the next is unclear.

Upon nuclear entry, HPV establishes low copy numbers (establishment) within the nucleus of the infected host cell [130]. The initial establishment phase is believed to require E1 and E2 and utilize the previously described mechanism of viral replication, whereby E2 initiates duplication of viral genomes through the recruitment of E1 to the viral ori [130, 131]. Following establishment the viral replicative program switches into the maintenance phase. During maintenance viral episomes are duplicated once per cell cycle [132] and are equally partitioned to daughter cells following division by E2 [81-84], thereby maintaining the established copy number per cell. As infected host cells become increasingly differentiated during the progression towards the outer epithelial layers viral replication switches to the amplification phase [133, 134]. This phase of the viral replicative program is characterized by the amplification of HPV genomes until roughly 1000 copies are obtained per cell and culminates in the packaging of genomes into infectious viral particles by L1 and L2 capsid proteins.

Amplification phase utilizes the host DNA damage response (DDR) pathway, which has been shown to be essential for completion of the viral replicative program [135]. HPV infection activates both ATM [136] and ATR [137, 138] DDR pathways. The primary DDR pathway believed to be involved in HPV replication is the homologous recombination DNA-damage response (HR-DDR) [139] (Figure 1.9). HR-DDR is a high fidelity, error-free method by which double-strand DNA breaks are repaired using an available homologous DNA template [140]. The cellular DDR kinase, ATM, is required for amplification phase replication occurring through the HR-DDR [136] and unsurprisingly, several HR-DDR proteins are essential for duplicating viral genomes during this phase, such as Rad51 [141, 142], SMC1 [143], BRCA1 and NBS1 [144]. However, precisely how the host HR-DDR is used during HPV replication remains an area of active research [139].

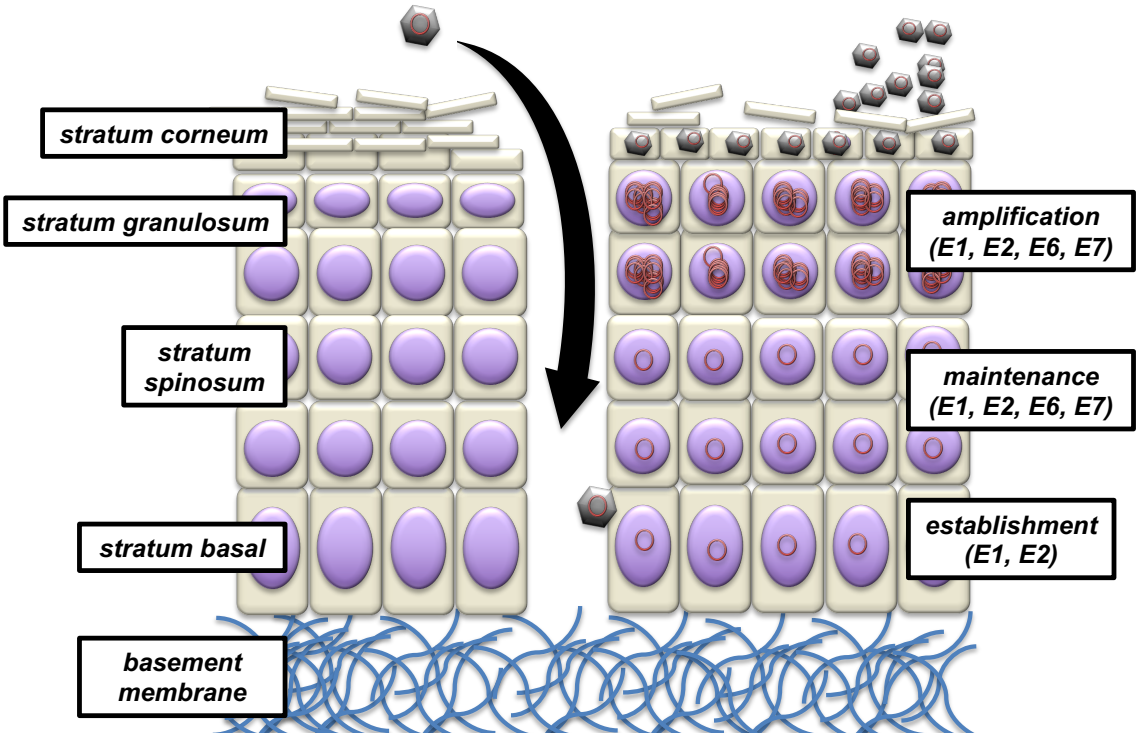

Figure 1.8. HPV life cycle. Following an insult to the epithelium HPV nucleocapsids infect basal keratinocytes and establish infection mediated by E1 and E2 proteins (establishment). The viral replicative program then enters maintenance phase and viral genome copy numbers are maintained dependent upon E1, E2, E6 and E7 proteins. Amplification phase occurs in the outer epithelial layers wherein viral genome copy numbers are amplified to thousands of copies per cell. HPV genomes are then packaged into infectious virions by L1 and L2 capsid proteins.

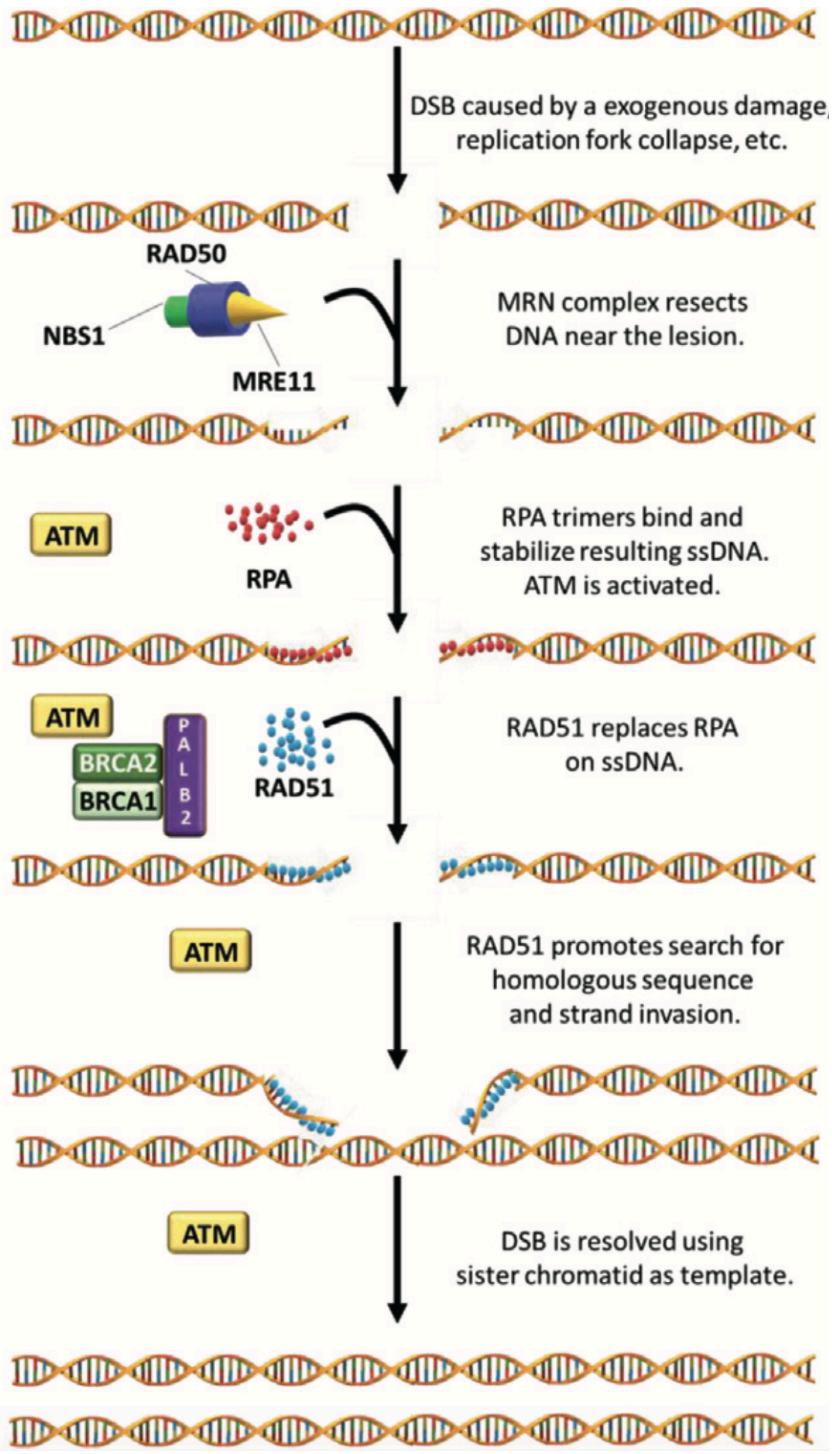

Figure 1.9. Homologous recombination DNA repair. HR-DDR is an error-free pathway used to correct double-strand DNA breaks. This figure was taken from [139].

1.4 Structural Maintenance of Chromosomes 5/6 (SMC5/6) Complex

SMC5/6 belongs to the evolutionarily conserved structural maintenance of chromosomes protein family, which includes SMC1/3 (cohesin) and SMC2/4 (condensin) [145]. Similar architectures of all SMC complexes can be found in both eukaryotes and prokaryotes, with complexes adopting a closed ring structure comprised of two core SMC heterodimers and multiple non-SMC subunits. All SMC complexes utilize ATP hydrolysis to facilitate SMC complex conformational changes, translocation along chromatin and the driving of topological changes in host chromatin [146]. Essential for development and cell viability, SMC5/6 is ubiquitously expressed in all tissues. SMC5/6 is localized within the nuclei of cells where it performs a variety of cellular functions that remain less well defined than its SMC1/3 and SMC2/4 counterparts. The primary functions of SMC5/6 are believed to be involved in the DNA damage response, where SMC5/6 has been shown to be essential to the functioning of the homologous recombination repair pathway used to mediate error-free correction of double-strand DNA breaks, as well as in restarting stalled replication forks [147]. However, precisely how SMC5/6 function in this pathway, or the full complement of its relevant substrates remain poorly defined.

1.4.1 Structure

The SMC5/6 complex is comprised of two SMC subunits SMC5 and SMC6, and four non-SMC elements (NSE1-4) which join to form a ring-shaped structure used to topologically entrap DNA [147] (Figure 1.10). Both SMC5 and SMC6 peptides fold back upon a central hinge region that connects to N- and C-terminal globular domains via a long coiled-coil alpha helical arm [148]. The N- and C-terminal globular domains contain Walker A and Walker B motifs respectively and provide the ATPase activity that is essential to opening and closing of the ring structure, binding DNA and translocation along chromatin. SMC5 and SMC6 then form heterodimers via their central hinge domains [148, 149]. The characteristic ring structure of the SMC5/6 complex is formed following the binding of the C-terminus of the NSE4 kleisin subunit with the distal portion of the SMC5 head domain and the base of the coiled-coil of SMC6 via its N-terminus [150]. In addition to closing the ring structure, NSE4 mediates the transient contact of SMC5/6 head domains to facilitate ATP hydrolysis. NSE1 and NSE3 bind NSE4 [148], forming the NSE1/3/4 sub-complex that enables NSE3-dependent DNA binding activity of SMC5/6 and mutations of key basic residues within the C-terminal DNA binding domain of NSE3 render SMC5/6 unable to bind DNA [151] (Figure 1.11). Intriguingly, while the NSE1 subunit of this sub-complex possesses a RING-like finger motif associated with E3 ubiquitin ligase activity, its function is not believed to be ubiquitination of substrates, but rather the assembly of the NSE1/3/4 trimer, as well as SMC5/6 stability and recruitment

to chromatin in response to DNA damage [152]. Amongst SMC protein family members, SMC5/6 is unique in possessing intrinsic enzymatic activity via an NSE2 SP-RING SUMO ligase subunit that is bound to the coiled-coil arm of SMC5 [153, 154]. While the full complement of NSE2 substrates have yet to be identified, NSE2, SMC5, SMC6, NSE3 and NSE4 have all been shown to be substrates for NSE2-mediated SUMOylation [155-158]. With the exception of NSE2, depletion of any one subunit results in SMC5/6 complex formation failure and proteasomal degradation [153].

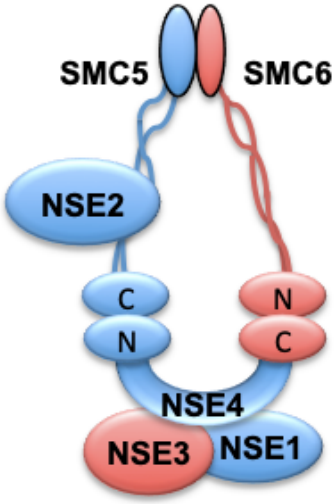

Figure 1.10. SMC5/6. Structural diagram of the *Homo sapiens* SMC5/6 complex. SMC5 and SMC6 form heterodimers via the subunits central hinge region and the complex ring is closed by kleisin subunit, NSE4. NSE1 and NSE3 bind NSE4 to form the NSE1/3/4 sub-complex. The NSE2 SUMO ligase is attached to the coiled-coil arm of SMC5.

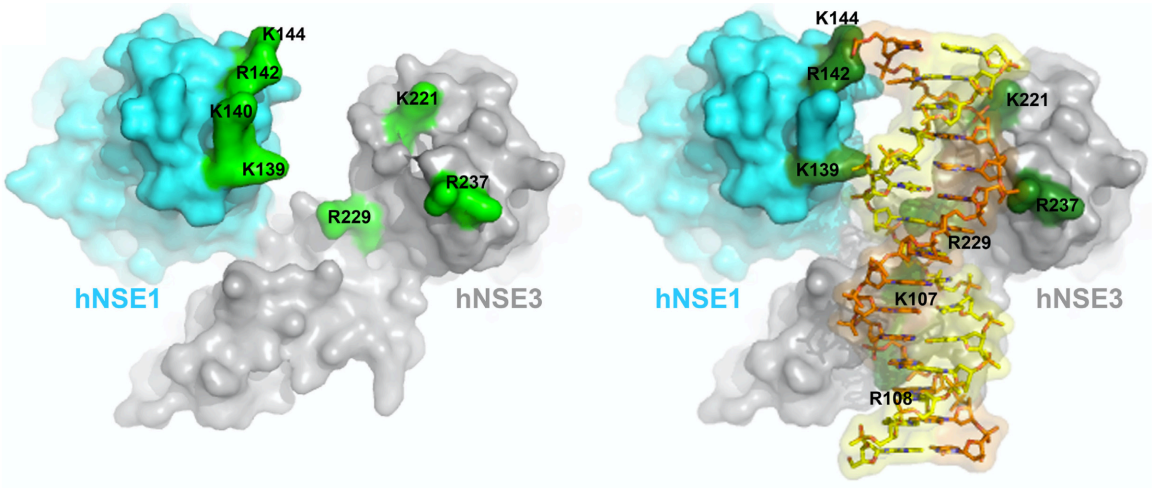

Figure 1.11. NSE1/3 sub-complex. (Left) Molecular surface representation of the NSE1/3 sub-complex (PDB ID: 3NW0) with key DNA binding basic residues highlighted in green. (Right) Postulated DNA binding pose of the NSE1/3 sub-complex with representative B-form DNA and phosphate contacting basic residues highlighted in green. This figure was taken from [151].

1.4.2 Recruitment and Localization

SMC5/6 associates with DNA during S phase of the cell cycle [159-161], but the majority of SMC5/6 is not present on DNA during mitosis [146]. Precisely how human SMC5/6 is localized to DNA and the factors involved in mediating this process remain to be fully understood. During the HR-DDR, SMC5/6 localization factor 1 and 2 (SLF1/2) have been proposed to recruit SMC5/6 to host chromatin during DNA damage in a Rad18-dependent manner [162]. In this model the coiled-coil arm of SMC6 is bound by the SLF2 subunit of the SLF1/2 heterodimer. RNF8-mediated mono-ubiquitination of γH2AX stimulates ubiquitin chain-elongation via RNF168 polyubiquitination. This is followed by SMC5/6 recruitment to sites of DNA damage by Rad18 binding γH2AX ubiquitin chains and SLF1. However, recent findings suggest that SMC5/6 localization to viral DNA and plasmids may differ than those facilitating its recruitment to damaged host DNA, and instead be mediated through an alternative pathway. SMC5/6 has been shown to bind episomal HBV covalently closed circular DNA (cccDNA) and HSV-1 DNA, but not be associated with integrated viral DNA [163, 164]. This preferential association with episomal viral DNA is postulated to involve the cellular E3 ligase, PJA1 [164]. In this model PJA1 replaces NSE1 in the NSE1/3/4 trimeric subcomplex and directs the SMC5/6 complex to episomal viral DNA, but how this occurs remains unclear.

1.4.3 Functions

Despite structural similarities to SMC1/3 and SMC2/4, the functions of SMC5/6 remain enigmatic [147]. SMC5/6 associates with host chromatin to rescue stalled replication forks [165], topologically entrap extrachromosomal DNA and mediate homologous recombination DNA repair (HR-DDR) [166], but precisely how SMC5/6-mediates these functions remains unclear [147]. The role of SMC5/6 in HR-DDR requires the complexes NSE2 SUMOylation activity, as loss of NSE2 renders cells hypersensitive to DNA damage [155, 167]. However, little is known about what substrates of NSE2 are required for this function [155].

Recently, antiviral roles have been described for SMC5/6 whereby SMC5/6 restricts viral DNA transcription of hepatitis B [163, 164, 168, 169] and herpes simplex-1 (HSV-1) viruses [164], but how SMC5/6 represses transcription of extrachromosomal DNA templates has yet to be determined. SMC5/6 reportedly interacts with both high, and low-risk HPV E2 proteins [170-172] and is purportedly involved in HPV genome maintenance [173], but the full extent of SMC5/6 functions during the life cycle of HR-HPV remain to be fully understood.

I examined interactions of SMC5/6 complex subunits SMC6 and NSE3 with HPV-31 E2 and E1 proteins, as well as their roles during the maintenance phase of the HPV-31 replicative program. Previously reported antiviral functions of SMC5/6 during HBV and HSV-1 infections led me to hypothesize that SMC5/6

would function as a repressor of the HPV-31 life cycle. I found that depletion of SMC6 and NSE3 resulted in increased HPV-31 replication and transcription, suggesting that the SMC5/6 complex represses the replicative program of HPV-31. My data demonstrate that SMC6 is present on episomal HPV-31 genomes and associates with E2 proteins but not E1/E2 replicative complexes. I find that SMC6 interactions with E2 are reduced in the presence of E1, implying that SMC6 competes with E1 for E2 binding. Taken together, these results indicate that SMC5/6 functions as a repressor of the HPV-31 replicative program potentially through inhibiting E1/E2 interactions.

CHAPTER 2

MATERIALS & METHODS

2.1 Plasmids, siRNA and Antibodies

Plasmids used were FLAG-SMC6/pcDNA3 (GenScript), HPV-31 FLAG-E2/pcDNA3, HPV-31 FLAG-E2mt (residues 205-372; [174]), HPV-31 V5-E2/pcDNA3, HPV-31 HA-E1/pcDNA3, and pcDNA3 (Table A.3). siRNA depletion of SMC5/6 subunits used esiGFP (Sigma-Aldrich; EHUEGFP), esiSMC6 (Sigma-Aldrich; EHU026931), siScramble DsiRNA (IDT; 51-01-19-08), siSMC6 (IDT; hs.Ri.SMC6.13.2) and siNSE3 (IDT; hs.Ri.NDNL2.13.1) (Table A.4). shRNA knockdowns used lentiviral transduction of either shGFP/pLKO.1 (Sigma-Aldrich; SHC002) or NSE3/pLKO.1 (Sigma-Aldrich; TRCN0000115778) constructs packaged in HEK293TT cells by co-transfection with PAX2 and MD2.G lentiviral packaging vectors. Antibodies included mouse M2 anti-FLAG (Sigma), mouse anti-V5 (Invitrogen), rabbit anti-V5 (Cell Signal Technologies), rabbit anti-NSE3 (Abcam), mouse anti-SMC6 (Abgent), rabbit anti-SMC6 (Bethyl), rat anti-HPV-31 E1 [175], mouse DM1a anti-α-tubulin (Sigma), mouse anti-β-actin (Sigma), mouse TVG-261 anti-HPV-16 E2 (Abcam), sheep anti-HPV-16 E2 [176], and control rabbit IgG (Jackson). Chromatin immunoprecipitation (ChIP) experiments used rabbit anti-HPV-31 E2 (10008, final bleed; [175]), rabbit anti-SMC6 (Bethyl), rat anti-HPV-31 E1 [175] and rabbit IgG (Jackson) antibodies. A complete list of antibodies can be found in Tables A.5.

2.2 Cell Culture

HEK293 and HEK293TT cells (J. Schiller and C. Buck) were maintained in Dulbecco's Modified Eagle Medium (Life-Technologies) supplemented with 10% fetal bovine serum (Atlas Biologicals) and 5% penicillin/streptomycin (Life-Technologies) at 37°C and 5% CO_2. CIN612-9E cells (L. Laimins) maintaining episomal HPV-31 genomes, were cultured in E-media with mitomycin C treated J2 3T3 fibroblast feeders (H. Green). CIN612-9E/shGFP and CIN612-9E/shNSE3 were cultured as parental CIN612-9E cells, but with 1µg/mL puromycin. CIN612-9Ei (L. Laimins) are derived from CIN612-9E cells, but possess integrated, not episomal, HPV-31 genomes and were maintained in E-media. N/TERT foreskin keratinocytes (N/TERT; I. Morgan), a cell line immortalized by hTERT expression, were cultured in Keratinocyte-SFM (Life-Technologies) supplemented with human recombinant EGF and bovine pituitary extract, as well as hygromycin B (4µg/mL) at 37°C and 5% CO_2. N/TERT/HPV-16 E2 were cultured as parental N/TERT cells. HaCaT/pcDNA3 and HaCaT/HPV-31 FLAG-E2 in Dulbecco's Modified Eagle Medium (Life-Technologies) supplemented with 10% fetal bovine serum (Atlas Biologicals) and 5% penicillin/streptomycin (Life-Technologies) with 200µg/mL G418 at 37°C and 5% CO_2. NIKS/HPV-31, a cell line maintaining episomal HPV-31, were cultured in F-media with 250µg/mL G418 and mitomycin C treated J2 3T3 fibroblast feeders. A complete list of cell lines used in these studies can be found in Table A.2.

E-media (Filter sterilize and store at 4°C)

- 750 mL DMEM
- 250 mL F-media (Ham's)
- 50 mL 100% FBS
- 24.3 mg adenine
- 1 mL 5 mg/mL insulin
- 1 mL 20 µg/mL EGF
- 2.5 µL cholera toxin
- 10 mL 100X Penicillin/Streptomycin (C_f = 1X)
- 100 µL 5 mg/mL hydrocortisone
- 1 mL 5 mg/mL transferrin

F-media (filter sterilize and store at 4°C)

- 750 mL F-media (Ham's)
- 250 mL DMEM
- 50 mL 100% FBS
- 24.3 mg adenine
- 1 mL 5 mg/mL insulin
- 2 mL 10 µg/mL EGF
- 2.5 µL cholera toxin
- 10 mL 100X Penicillin/Streptomycin (C_f = 1X)
- 100 µL 5 mg/mL hydrocortisone

2.3 Co-immunoprecipitation and Immunoblotting

HEK293 and HEK293TT cells were transfected using polyethylenimine (PEI; 2mg/mL) (Sigma) per the manufacturer's protocol. Cells were seeded onto 10cm plates and transfected, then after 48hrs cells were lysed in 0.5% NP-40/150mM NaCl/50mM Tris-HCl (pH 8)/10% glycerol and protease inhibitor cocktail (Sigma). Samples were then rotated for 1hr at 4^{o}C after the addition of 4µL benzonase (Millipore), then spun for 10min at 4^{o}C and supernatant collected. FLAG immunoprecipitation experiments had samples rotated at 4^{o}C with 20µL of 50% M2 agarose bead slurry (Sigma). SMC6 immunoprecipitation experiments used rabbit anti-SMC6 antibody. Beads were then washed 4 times, alternating 0.5% NP-40/0.5M NaCl/50mM Tris-HCl (pH 8)/10% glycerol and 0.5% NP-40/150mM NaCl/50mM Tris-HCl (pH 8)/10% glycerol washes, then boiled for 3-5min at 95^{o}C. Samples were subjected to SDS-PAGE and subsequent Western blot analysis using a 4-12% Bis-Tris gradient gel (GenScript), then transferred onto PVDF membranes (Millipore) using semi-dry transfer. PVDF membranes were blocked in 5% milk/1X PBS/Tween-20 (0.1%), then probed rocking overnight at 4^{o}C with primary antibodies. Next, antibody detection was performed using appropriate secondary antibodies with chemiluminescence substrates (GE) and ImageQuant software.

2.4 Protein Isolation

To analyze the influence of HPV E2 on SMC6 stability total protein was isolated from cells stably expressing either HPV-31 (HaCaT) or HPV-16 (N/TERT). Cells were lysed in 10% SDS/150 mM NaCl lysis buffer lysis buffer and protease inhibitor cocktail, then Laemmli buffer added and samples boiled. SDS-PAGE was performed using a 4-12% Bis-Tris gradient gel (GenScript) and Western blot analysis performed as described for co-immunoprecipitation experiments. Densitometry was performed using ImageStudios software and SMC6 protein expression normalized relative to loading controls. Statistical significance was determined using a two-tailed Student's T-test. To confirm depletion of SMC6 and NSE3 in RNAi knockdown experiments, total protein was isolation and Western blot analysis was performed as described above.

10% SDS/150 mM NaCl lysis buffer [177]:

- 333 µL Tris pH 8.0 (C_f = 10 mM)
- 1.5 mL 5 M NaCl (C_f = 150 mM)
- 10 mL 10 % SDS (C_f = 2 %)
- 38.2 mL MQ-H_2O

2.5 qPCR DNA Replication Assay

Transient depletion of SMC5/6 subunits was performed in CIN612-9E and NIKS/HPV-31 cells reverse-transfected with 15nM or 20nM siRNA, respectively, using Lipofectamine 2000 (Life-Technologies) per the manufacturer's protocol. The following day media was changed and cells returned to incubate for an additional 24 hours. DNA isolation was performed by phenol/chloroform extraction as previously described [178]. Purification of DNA was performed by ethanol precipitation. Briefly, DNA samples were mixed with 100% ethanol and 3M sodium acetate at a ratio of 1:2:0.01, then incubated at -20^{o}C for 1 hour (or overnight) to precipitate DNA. Samples were then spun at 4^{o}C for 1 hour to pellet DNA, then supernatant removed and residual ethanol allowed to evaporate at room temperature. DNA pellets were then resuspended in either MQ-H_2O or TE. qPCR was performed as described above with C_t values normalized to β-actin and fold-change calculated relative to control groups. All experiments were performed in triplicate and statistical significance determined by either two-tailed (CIN612-9E), or one-tailed (NIKS/HPV-31) Student's T-test.

qPCR cycling conditions

- Initial Denaturation: 95^{o}C x3 min
- Denaturation: 95^{o}C x10 sec
- Annealing/Elongation: 60^{o}C x30 sec
- Melt Curve: 60-95^{o}C, 0.5^{o}C increments
- Cycles: 40

2.6 RT-qPCR

RNA was isolated using TRIzol reagent and PureLink RNA Micro kits (Invitrogen) per the manufacturer's protocol. RQ1 DNase (Promega) was used per the manufacturer's protocol to remove any possible genomic DNA contaminants. Reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen) per the manufacturer's protocol, then qPCR performed as described above with C_t values normalized to GAPDH, or β-actin, fold-change calculated relative to control groups and primers listed in Table A.6. All experiments were performed in triplicate and statistical significance determined by either two-tailed (CIN612-9E), or one-tailed (NIKS/HPV-31) Student's T-test.

RT-PCR cycling conditions

- Cycle 1 25°C x10 min
- Cycle 2 50°C x50 min
- Cycle 3 70°C x15 min
- Cycle 4 12°C x ∞
- Cycles x40 (Cycle 1-3)

2.7 Site-directed Mutagenesis

Replication defective HPV-16 genomes were generated by site-directed mutagenesis (SDM) for future studies examining the role of SMC5/6 as a transcriptional repressor of HPV. Briefly, a translation termination linker (TTL) encoding 3 sequential stop codons was used to mutate the viral E1 open reading frame using PCR. The site selected for placement of the E1 TTL would mutate nucleotides encoding E1 amino acids 149-151 and remove a KasI restriction endonuclease site within E1 ORF of HPV-16/pMC (Figure 2.1), but not disrupt E1^E4 or E8^E2 (Figure 2.2). Removal of the KasI site was used for screening of candidate bacterial colonies using restriction digest and agarose gel electrophoresis. SDM PCR reactions were set up using HPV-16/pMC as follows:

SDM Reaction:

- 100 ng HPV-16/pMC
- 10.0 µL 5X Phusion Buffer
- 1.0 µL 10 µM dNTP
- 1.0 µL E1 TTL primer
- 1.0 µL Phusion polymerase
- X µL MQ-H_2O (V_f = 50 µL)

PCR cycling conditions

- Initial Denaturation: 98^{o} C *x1 min*
- Denaturation: 98^{o} C *x15 sec*
- Annealing 60^{o} C *x15 sec*
- Elongation: 72^{o} C *x8-12 min*
- Repeat to 2: 20 cycles
- Final Elongation: 72^{o} C *x5 min*
- Final: 12^{o} C *x* ∞

PCR products were digested with 1 µL DpnI (NEB) at 37^{o}C to remove unmodified template DNA, then DpnI heated inactivated at 80^{o}C for 20 minutes. Bacteria were then transformed, selected, and plasmid DNA isolated using Pureyield Plasmid Miniprep (Promega) per the manufacturer's specifications. DNA was then digested with KasI and run on agarose gels to screen for potential colonies containing the desired mutation. Candidate colonies were then sequenced to confirm genomes possessed the desired mutations within E1.

HPV-16 E1^{TTL} Primer (F): 5'-atgttacaggtagaaTGATAATGAgagactgaaacacca-3'

HPV-16 E1 Primer (R): 5'-tgtctttcactaacaccctc-3'

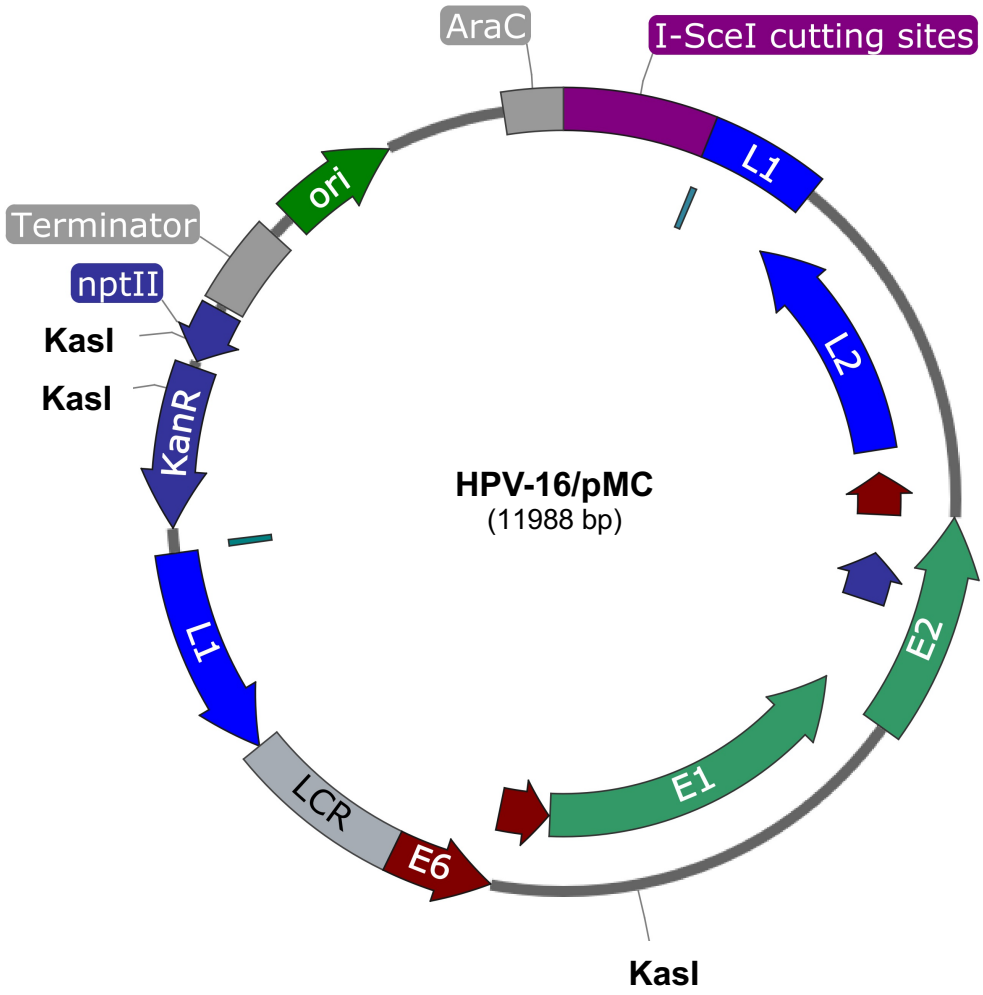

Figure 2.1. HPV-16/pMC. HPV-16 genomes in the pMC backbone were selected for use in inserting a translation termination linker (TTL) into the E1 ORF using site-directed mutagenesis. Successful insertion of the TTL into E1 would remove the KasI site within the E1 ORF and enable restriction digest screening of candidate colonies.

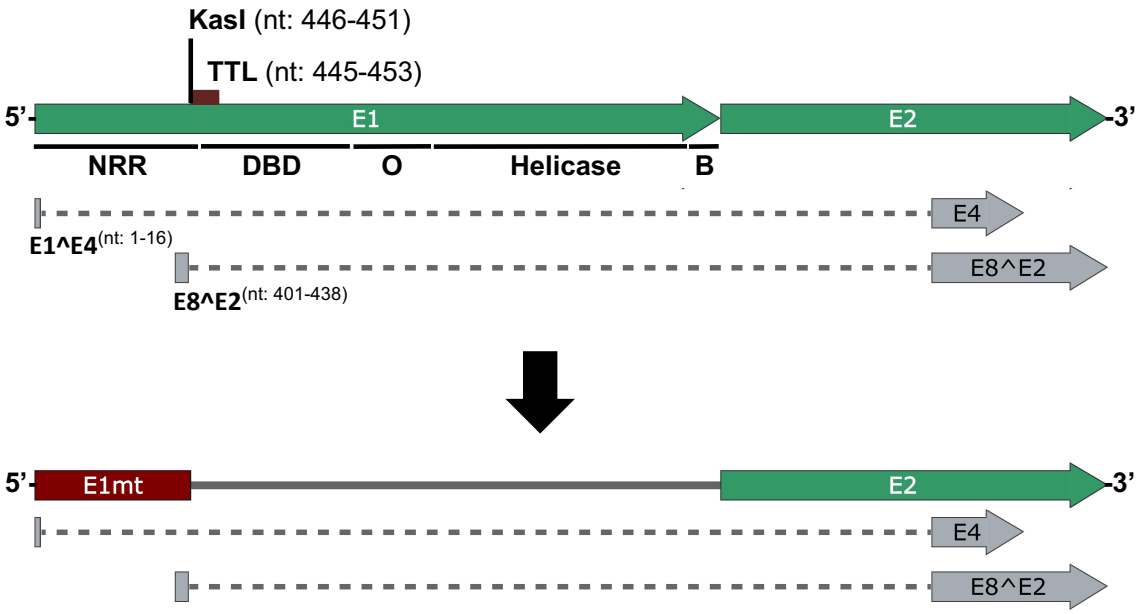

Figure 2.2. HPV-16 E1 mutant site-directed mutagenesis. (**Top**) Wild-type HPV-16 E1 possesses an N-terminal regulatory region (NRR), DNA-binding domain (DBD), oligomerization domain (O), helicase domain (Helicase) and C-terminal brace (B). E1^E4 and E8^E2 proteins are produced from spliced transcripts that include short peptides within the E1 ORF. (**Bottom**) HPV-16 genomes would have a translation termination linker (TTL – 3 sequential stop codons; UGA, UAA, UGA) placed within the viral E1 ORF after the codon for amino acid 148 and mutate G149, R150 and H151 to stop codons. E1 TTL insertion was downstream of E1^E4 (E1 nt: 1-16) and E8^E2 (E1 nt: 401-438) nucleotide sequences within E1.

2.8 Chromatin Immunoprecipitation

ChIP (Figure 2.3) was performed using CIN612-9E cells and ChIP-IT Express Enzymatic kit (Active Motif) according to the manufacturer's protocols. qPCR was performed using SsoFast Evagreen Mastermix (Bio-Rad) with primers specific to sequences within the HPV-31 L1 region, E2BS in the 5' region of the LCR, and E2BS located adjacent to the viral ori and early promoter (Figure 2.4). Primer sequences are listed in Table A.6. Data were analyzed using BioRad CFX with C_t values normalized to input samples and fold change calculated relative to control IgG samples (set to 1). Statistical significance was determined by comparison of fold change values against negative DNA binding sequences or control IgG using one-tailed Student's T-test.

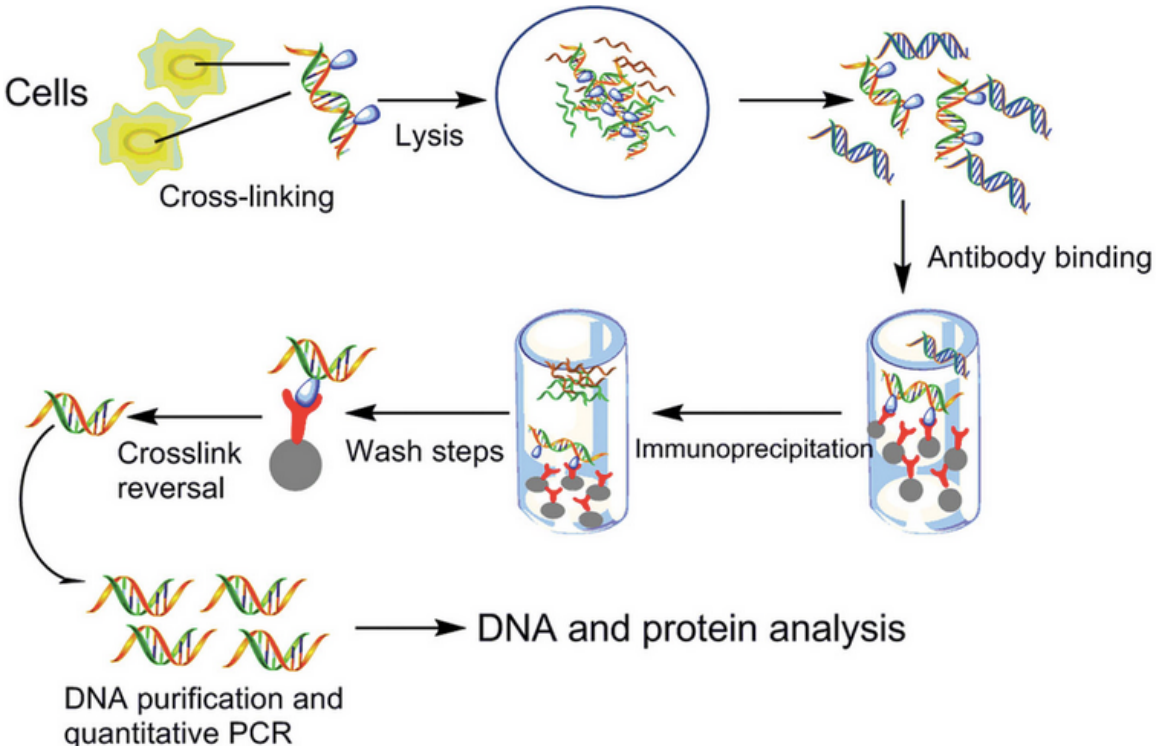

Figure 2.3. Chromatin immunoprecipitation. Diagram of chromatin immunoprecipitation “ChIP” assay. This figure was taken from [179].

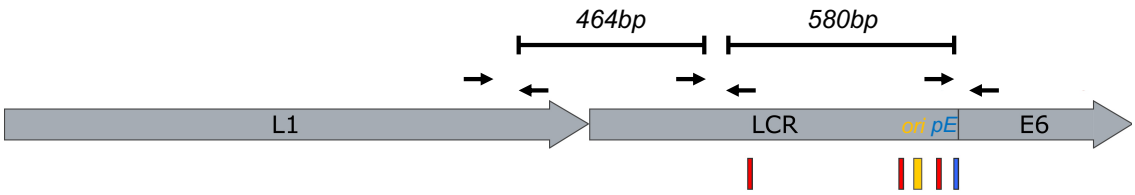

Figure 2.4. HPV-31 ChIP sites. CIN612-9E cells were tested for the presence of E2 and SMC6 on the viral LCR and flanking L1 region. Primers to the viral L1 region generated an amplicon roughly 464bp from the 5' upstream E2 binding site (E2BS - red) of the LCR. The 5' E2BS amplicon was approximately 580bp upstream of the 3' E2BS that was located adjacent to the viral ori (yellow) and early promoter (blue). Primer annealing sites are indicated by black arrows (right facing – forward primer; left facing – reverse primer).

CHAPTER 3

RESULTS

3.1 SMC6 and NSE3 Interactions with HPV-31/-16 E2

The SMC5/6 complex (Figure 3.1A) subunit SMC6 reportedly co-precipitates with several HPV E2 proteins (Figure 3.1B) [170-172] so I began this investigation by attempting to validate this interaction with HPV-31. HEK293 cells were transfected with either FLAG-SMC6, HPV-31 V5-E2, or both plasmids, then FLAG-SMC6 immunoprecipitation performed using M2 anti-FLAG antibody. Both HPV-31 V5-E2 and NSE3 (positive control) co-immunoprecipitated (co-IP) with SMC6 (Figure 3.1C). In the reciprocal experiment, antibody to the V5 epitope pulled-down FLAG-SMC6 but not the SMC5/6 subunit NSE3 (Figure 3.1D). Next, I sought to confirm an interaction of SMC6 with HPV-16 E2. HEK293TT cells were transfected with FLAG-SMC6, HPV-16 FLAG-E2, or both plasmids and then IP performed using anti-SMC6 antibody. I observed SMC6 co-IP NSE3 and HPV-16 FLAG-E2 when co-expressed with FLAG-SMC6 (Figure 3.2). These data support that SMC6 interacts with both HPV-31 and HPV-16 E2 proteins. This shows that NSE3 does not associate with HPV-31 E2, suggesting that E2 may disrupt SMC6 interactions with NSE3.

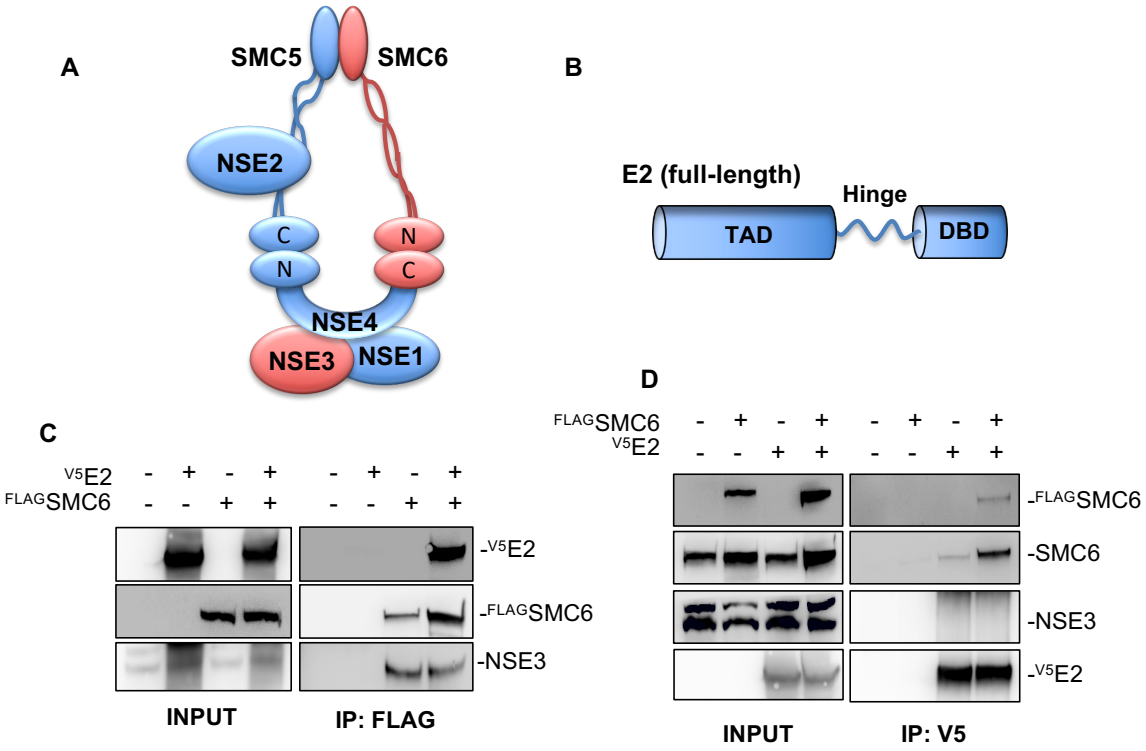

Figure 3.1. SMC6 co-immunoprecipitates HPV-31 E2. (**A**) Figure representation of the SMC5/6 complex with subunits investigated in red. (**B**) Figure representation of HPV-31 full-length E2. (**C**) V5-tagged HPV-31 E2 and FLAG-SMC6 were transfected (+) into HEK293 cells. Forty-eight hours later FLAG was immunoprecipitated and immunoblotting performed using rabbit anti-V5, mouse M2 anti-FLAG and rabbit anti-NSE3 antibodies. (**D**) As in C,but using HEK293TT cells and IP using rabbit anti-V5 antibody. Immunoblotting was performed using rabbit anti-V5, mouse M2 anti-FLAG, rabbit anti-SMC6 and rabbit anti-NSE3 antibodies.

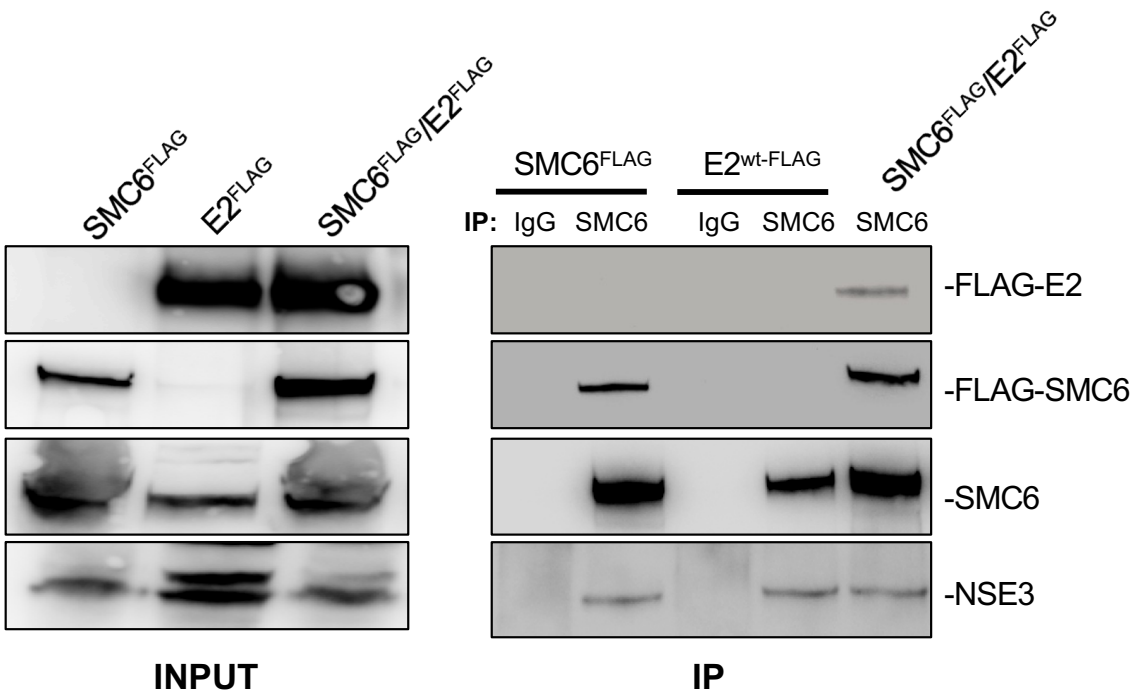

Figure 3.2. FLAG-SMC6 co-immunoprecipitates HPV-16 E2. FLAG-tagged HPV-16 E2 and FLAG-SMC6 were transfected into HEK293TT cells. Forty-eight hours later immunoprecipitation was performed using either rabbit IgG or rabbit anti-SMC6 antibodies, then immunoblotting performed using mouse M2 anti-FLAG, rabbit anti-SMC6 and rabbit anti-NSE3 (positive control) antibodies.

Structurally, HPV E2 is comprised of an N-terminal transactivation domain (TAD) connected to a C-terminal DNA binding/dimerization domain (DBD) by a poorly conserved linker region. To identify the region of E2 required for these interactions, HEK293TT cells were transfected with FLAG-SMC6, FLAG-tagged full-length E2, or FLAG-tagged truncated E2 expressing the E2 hinge and DBD (Figure 3.3A). Co-IP was performed using anti-SMC6 antibody and showed full-length HPV-31 FLAG-E2 and NSE3 co-IP with SMC6, but not truncated E2 (Figure 3.3B). These data demonstrate that SMC6 association with HPV-31 E2 requires the E2 transactivation domain, inferring E2 interacts with the full-length E2 isoform.

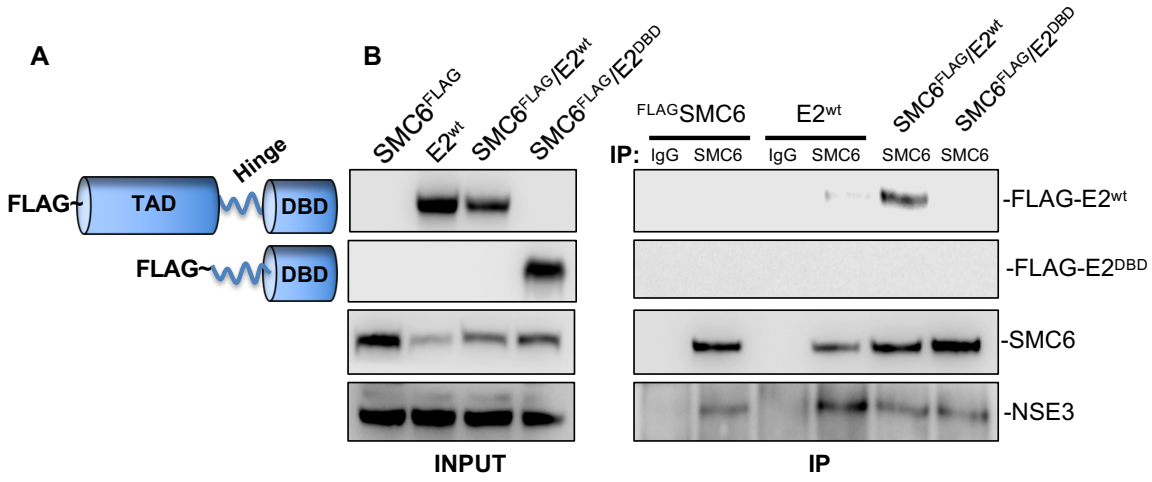

Figure 3.3. SMC6 co-immunoprecipitates with full-length HPV-31 E2. (**A**) Figure representation of FLAG-tagged E2 proteins used to identify region of SMC6 interaction. (**B**) FLAG-SMC6, full-length FLAG-tagged HPV-31 E2 and FLAG-tagged HPV-31 truncated E2 mutant were transfected into HEK293TT cells. Forty-eight hours later immunoprecipitation was performed using either rabbit IgG or rabbit anti-SMC6 antibodies, then immunoblotting performed using mouse M2 anti-FLAG, rabbit anti-SMC6 and rabbit anti-NSE3 (positive control) antibodies.

3.2 SMC5/6 Complex and HPV-31 Replication/Transcription

SMC5/6 purportedly inhibits the life-cycle of DNA viruses by restricting viral transcription in studies of both hepatitis B [163, 164, 180, 181] and herpes simplex-1 viruses [164]. I questioned if the previously described antiviral functions of SMC6 and its interactions with HPV E2 indicated that SMC5/6 functions as a repressor of HPV-31 replication or transcription. I began investigating this by depleting CIN612-9E cells of SMC6 using siRNA knockdown, then examining HPV-31 DNA content and transcript abundance. CIN612-9E cells transfected with siRNA targeting SMC6 had reduced SMC6 protein (Figure 3.4A) and transcript levels (Figure 3.4B) compared to those transfected with control siRNA. Indeed, SMC6 depletion resulted in increased viral DNA content relative to the control group (Figure 3.4C). Similarly, RT-qPCR analysis revealed that SMC6 depletion resulted in increased HPV-31 E2 and E1 transcript abundance (Figure 3.4B). I sought to further validate these findings by examining the effects of depleting both SMC6 and SMC5/6 subunit, NSE3 in CIN612-9E cells by siRNA knockdown (Figure 3.5A). SMC6 and NSE3 depleted cells displayed increased HPV-31 DNA content (Figure 3.5B) and viral E8^E2 transcript abundance (Figure 3.5C). To confirm these findings, I performed an analogous experiment in episomal maintaining NIKS/HPV-31 keratinocytes. Similar results were found in that depletion of either SMC6 or NSE3 resulted in increased HPV-31 DNA content, but only NSE3 knockdown increased E8^E2 viral transcripts relative to controls in NIKS/HPV-31 (Figure 3.5D-F). To

determine if stable NSE3 depletion increased HPV-31 DNA content, CIN612-9E cells were transduced with lentivirus containing constructs with either non-targeting shRNA, or shRNA targeting NSE3 (Figure 3.6A). Unexpectedly, stable depletion of NSE3 did not influence viral replication relative to controls when HPV-31 DNA content was analyzed by qPCR (Figure 3.6B). Taken together, these data show that transient depletion of SMC6 and NSE3 subunits in CIN612-9E and NIKS/HPV-31 results in increased HPV-31 replication and transcription (Table 3.1), implying that SMC6 and NSE3 are repressors of the HPV-31 replicative program.

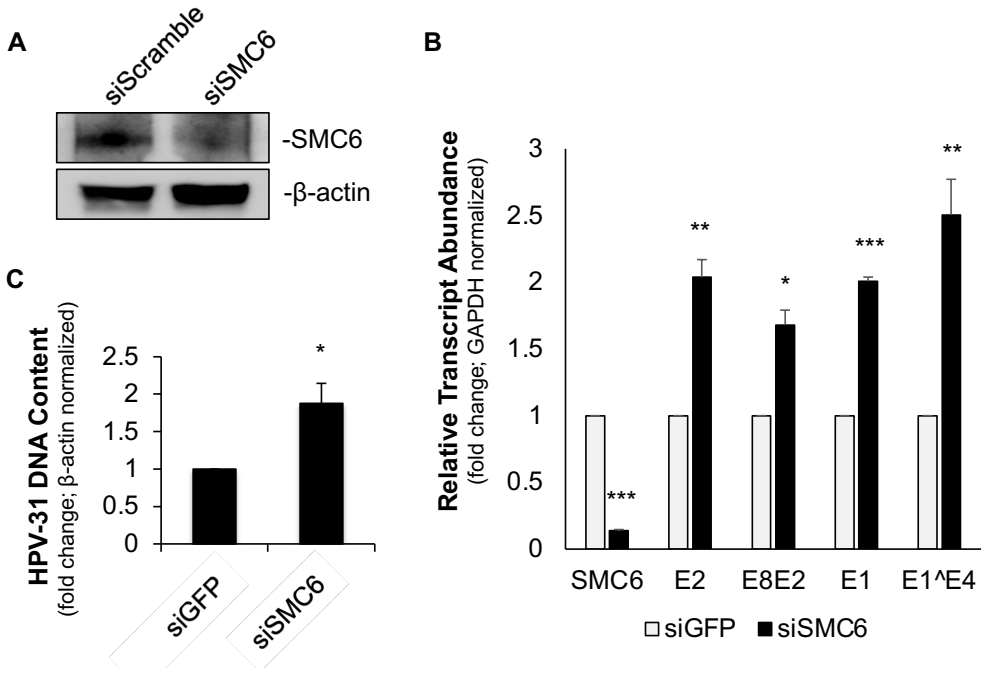

Figure 3.4. SMC6 inhibits HPV-31 replication and transcription. (**A**) Forty-eight hour siRNA knockdown of SMC6 resulted in decreased SMC6 expression in CIN612-9E cells. (**B**) Transient depletion of SMC6 increased HPV-31 DNA content in CIN612-9E cells following 48hr siRNA knockdown. C_t values were normalized to B-actin and fold change shown relative to control group (set to 1; n=3). (**C**) As in B, with RNA transcript abundance C_t values normalized to GAPDH and fold change shown relative to control group (set to 1; n=3). Western blot analysis was performed once to confirm knockdown, DNA and RNA experiments were performed in triplicate. Two-tailed Student's T-test was used to determine statistical significance where * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$ and # = $p<0.10$.

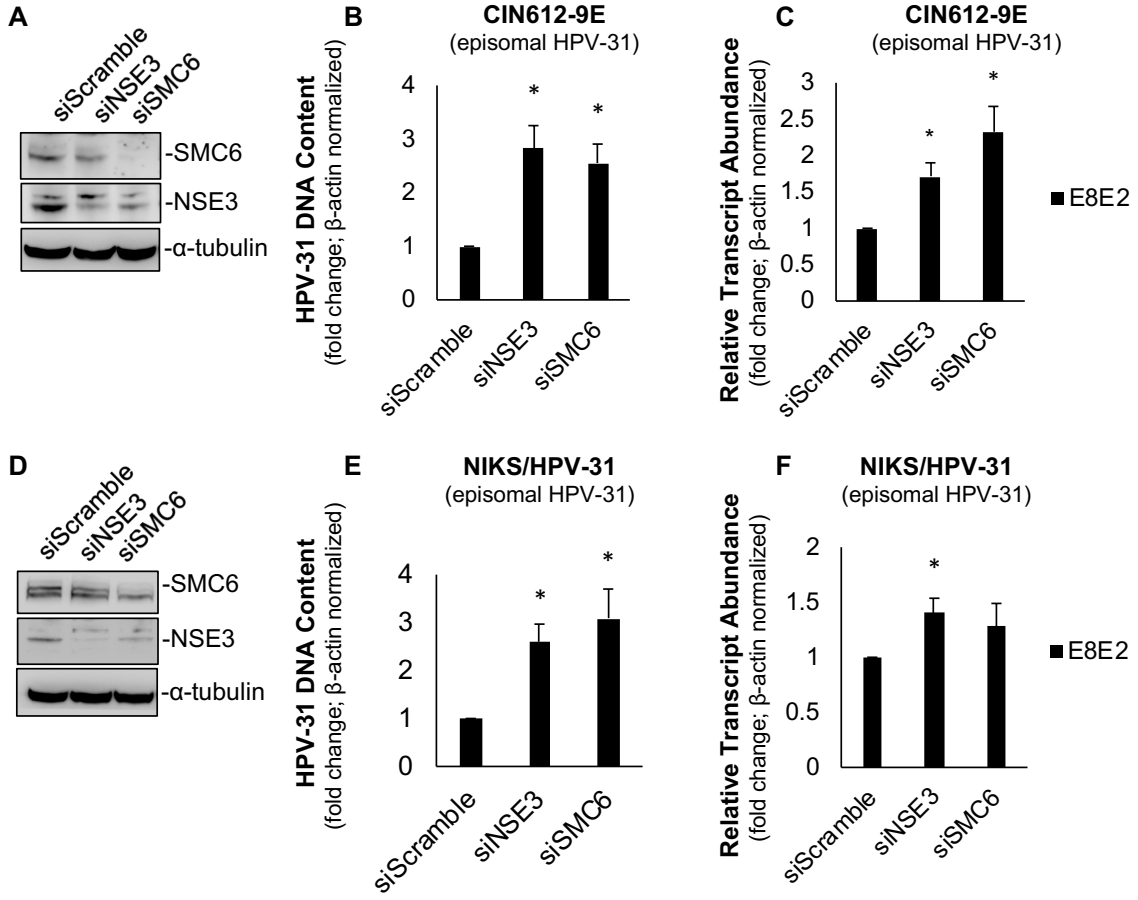

Figure 3.5. SMC6 and NSE3 repress HPV-31 replication and transcription in CIN612-9E and NIKS/HPV-31 cells. (**A**) Forty-eight hour siRNA knockdown of SMC6 and NSE3 resulted in decreased target protein abundance in CIN612-9E cells. (**B**) Transient depletion of SMC6 increased HPV-31 DNA content in CIN612-9E cells following 48hr siRNA knockdown. C_t values were normalized to β-actin and fold change shown relative to control group (set to 1; n=3). (**C**) As in B, but RNA transcript abundance C_t values normalized to β-actin and fold change shown relative to control group (set to 1; n=3). (**D**) As in A, with NIKS/HPV-31 cells. (**E**) As in B, with NIKS/HPV-31 cells. (**F**) As in C, with NIKS/HPV-31 cells. All experiments were performed in triplicate and statistical significance determined by two-tailed (CIN612-9E) or one-tailed (NIKS/HPV-31) Student's T-test where * = $p < 0.05$.

Table 3.1. SMC6 and NSE3 siRNA knockdown summary. Increased replication or transcription indicated by ' ⇧ ' and no change indicated by '-/- '.

Cell Line	siRNA	Replication	Transcription
CIN612	siNSE3	⇧	⇧
	siSMC6	⇧	⇧
NIKS/31	siNSE3	⇧	⇧
	siSMC6	⇧	**-/-**

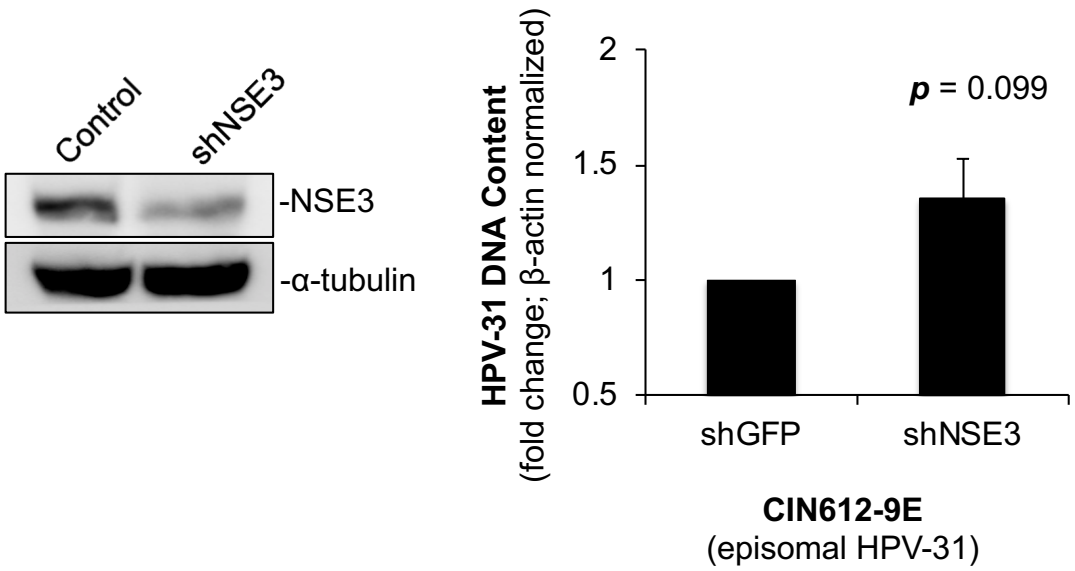

Figure 3.6. Stable NSE3 depletion does not influence HPV-31 replication in CIN612-9E cells. (**A**) shRNA knockdown of NSE3 resulted in decreased target protein abundance in CIN612-9E cells. (**B**) NSE3 depletion did not influence HPV-31 DNA content in CIN612-9E cells. C_t values were normalized to β-actin and fold change shown relative to control group (set to 1; n=3). All experiments were performed in triplicate and statistical significance determined by two-tailed (CIN612-9E) Student's T-test.

3.3 SMC6 Associates with HPV-31 Episomes

SMC6 associates with nuclear episomal hepatitis B virus (HBV) cccDNA [163, 164] and HSV-1 DNA [164], but how SMC5/6 is localized to viral DNA remains unclear. SMC6 association with HPV genomes has not been investigated. Similar to HBV cccDNA and HSV-1, HPV genomes are maintained as episomes adjacent PML nuclear bodies [182], known sites of SMC5/6 localization [181]. The association of SMC6 with viral DNA and its interaction with HPV E2 led us to question whether SMC6 is present on episomal HPV-31 genomes. To answer this, I first validated my ability to immunoprecipitate SMC6 in episomal HPV-31 maintaining CIN612-9E cells to determine suitability of the cell line for subsequent chromatin immunoprecipitation (ChIP) experiments. Total protein was isolated from CIN612-9E cells and SMC6 IP performed, followed by immunoblotting using anti-SMC6 antibody. SMC6 was detectable in input samples and IP samples where anti-SMC6 antibody was used, but not negative control rabbit IgG (Figure 3.7A). Next, double-thymidine treatment was used to synchronize the cell cycle of CIN612-9E cells in S phase and ChIP performed using either HPV-31 E2, SMC6 or rabbit IgG antibodies. qPCR determined that HPV-31 E2 was found E2BS within the HPV-31 LCR but not viral L1 region (Figure 3.7C). I found SMC6 was not localized to the L1 region but was present at the E2BS located within the 5' end of the LCR and E2BS proximal to the viral ori and promoter (Figure 3.7D). E2 has been shown to be present on host DNA [81, 183, 184] and its consensus sequences are found throughout the host

genome [183]. I examined whether SMC6 and E2 were present at a known host replication origin (GM-CSF) as a comparator for association with viral DNA. HPV-31 E2 but not SMC6 was present at the eukaryotic GM-CSF origin of replication (Figure 3.7B). These data show that SMC6 is present on HPV-31 episomes at E2 binding sites located within the LCR at the 5' region and near the viral ori and early promoter, but not flanking L1 region or eukaryotic GM-CSF origin of replication.

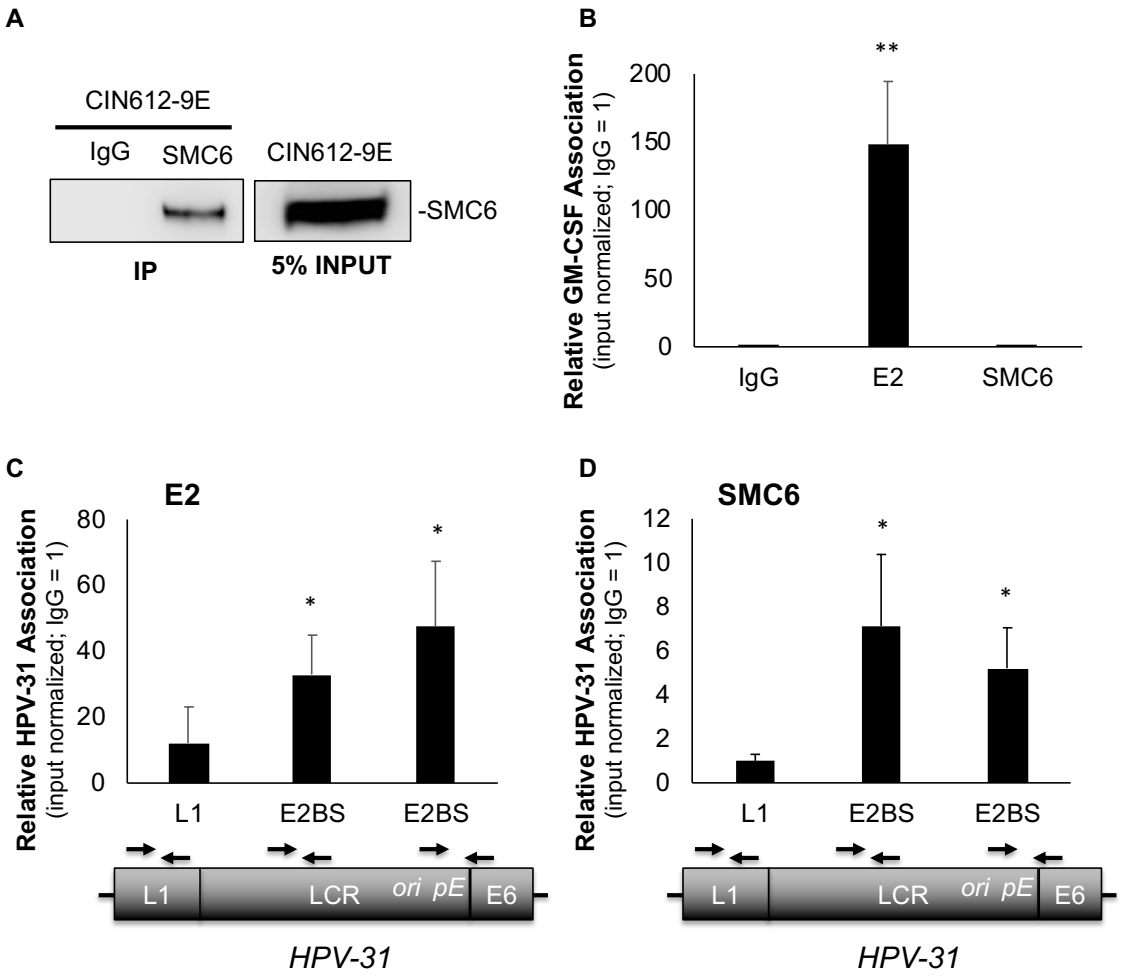

Figure 3.7. SMC6 is present on HPV-31 episomes in CIN612-9E cells. (**A**) Total protein was isolated from CIN612-9E cells and immunoprecipitation performed overnight using either rabbit anti-SMC6 or control rabbit IgG antibodies, then immunoblotting performed using rabbit anti-SMC6 antibody. (**B**) CIN612-9E cells were synchronized in G1/S phase by double-thymidine block and ChIP performed using rabbit anti-HPV-31 E2, rabbit anti-SMC6 or control rabbit IgG antibodies. qPCR was conducted using primers to the eukaryotic GM-CSF origin of replication with primers listed in Table A.6. (**C**) As in B, but examining E2 presence at viral L1, 5' upstream LCR E2 binding site, and E2BS proximal to the viral ori or early promoter (pE) sequences of the HPV-31 genome with approximate viral genome location and corresponding primers shown below their respective sites on the horizontal axis. (**D**). As in C, but with SMC6. C_t values were normalized to input with control IgG set to 1 and values expressed as mean fold change over IgG +/- SEM. ChIP was performed at least 4 independent times for all locations examined and statistical significance determined by one-tailed Student's T-test as compared to control IgG with * $p < 0.05$ and ** $p < 0.005$.

3.4 SMC6/NSE3 Interactions with HPV-31 E1 and E1/E2 Complexes

HPV replication depends on E2-mediated recruitment of the viral E1 DNA helicase to the viral origin of replication, for which a transient E1/E2-DNA ternary complex is formed [46]. The association of SMC6 with E2 and HPV-31 genomes, as well as its repression of viral replication, led us to speculate that SMC6 may interact with HPV-31 E1/E2 replicative complexes. To explore this, HEK293TT cells were transfected with FLAG-SMC6, HPV-31 V5-E2, HPV-31 HA-E1 plasmids alone or in combination and co-IP reactions performed using anti-SMC6 antibody. I observed HPV-31 V5-E2 and NSE3 co-IP with SMC6 (Figure 3.8A), not HPV-31 HA-E1 when E1 was expressed alone, co-expressed with E2, or when FLAG-SMC6 was expressed with E1 and E2 co-expression. Co-expression of E1 with FLAG-SMC6 and E2 did not affect SMC6 co-IP of NSE3 but reduced the amount of E2 pulled-down by SMC6. To confirm that SMC6 does not co-IP HPV-31 E1/E2 complexes or E1 alone, I performed an analogous experiment using anti-HPV-31 E1 antibody for co-IP. IP of HPV-31 HA-E1 pulled-down HPV-31 V5-E2 but not SMC6 or NSE3 (Figure 3.8B). E1 co-IP of E2 was not influenced by overexpression of FLAG-SMC6. Together these data demonstrate that neither SMC6 nor NSE3 associate with HPV-31 E1 or E1/2 complexes. Additionally, data show that expression of E1 during SMC6 IP does not affect SMC6 co-IP of NSE3 but inhibits SMC6 pull-down of E2, indicating the E1 competes with SMC6 for binding of E2.

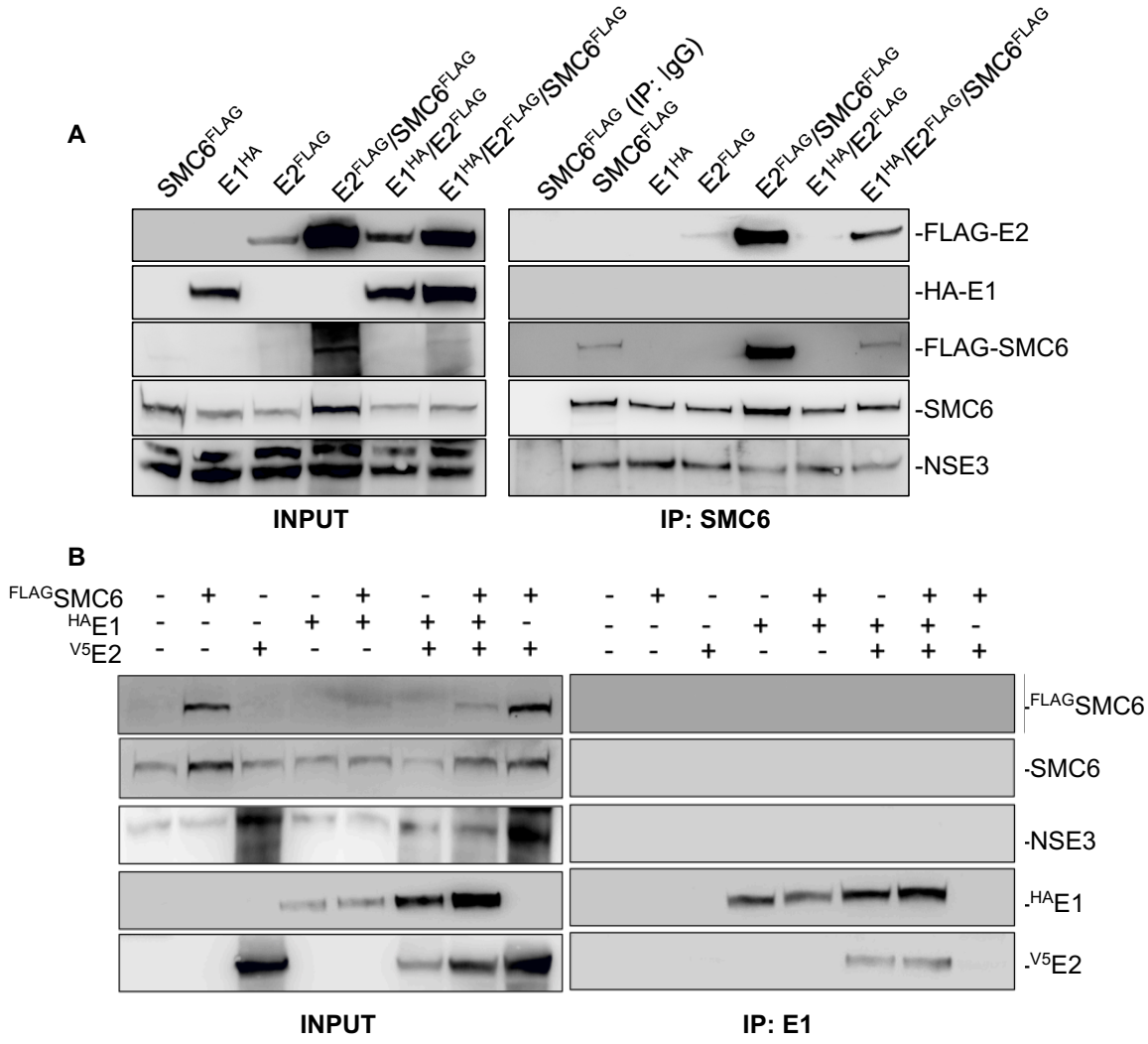

Figure 3.8. SMC6 and NSE3 do not co-immunoprecipitate with HPV-31 E2/E1 complexes or HPV-31 HA-E1 in HEK293TT cells. (**A**) FLAG-SMC6, HPV-31 V5-E2 and HPV-31 HA-E1 were transfected into HEK293TT cells. Forty-eight hours later immunoprecipitation was performed using either rabbit IgG or rabbit anti-SMC6 antibodies, then immunoblotting performed using anti-SMC6, rabbit anti-NSE3, mouse M2 anti-FLAG, or rat anti-HPV-31 E1 antibodies. (**B**) FLAG-SMC6, HPV-31 V5-E2 and HPV-31 HA-E1 were transfected (+) into HEK293TT cells. Forty-eight hours later immunoprecipitation was performed using rat anti-HPV-31 E1, then immunoblotting performed using rabbit anti-SMC6, rabbit anti-NSE3, mouse M2 anti-FLAG, rabbit anti-V5, or rat anti-HPV-31 E1 antibodies.

3.5 HPV-31/-16 E2 Effects on SMC6 Protein Levels

HPV E2 proteins influence host factor stability and may modify the transcriptional activity of host genes [79, 185]. Having validated E2/SMC6 interactions led us to question if E2 influenced SMC6 abundance. To explore this, I generated HaCaT cells stably expressing HPV-31 FLAG-E2 or control vector by G418 selection (Figure 3.9A). No changes in SMC6 protein levels in response to HPV-31 E2 expression were observed. To confirm this in another cell model I examined SMC6 protein expression in parental N/TERT cells and N/TERT/E2 cells stably expressing the HPV-16 E2 protein (Figure 3.9B). Similarly, no effect on SMC6 stability was found in N/TERT cells with HPV-16 E2 as compared to control cells. These data indicate that HPV-31 and HPV-16 E2 proteins do not affect SMC6 protein levels.

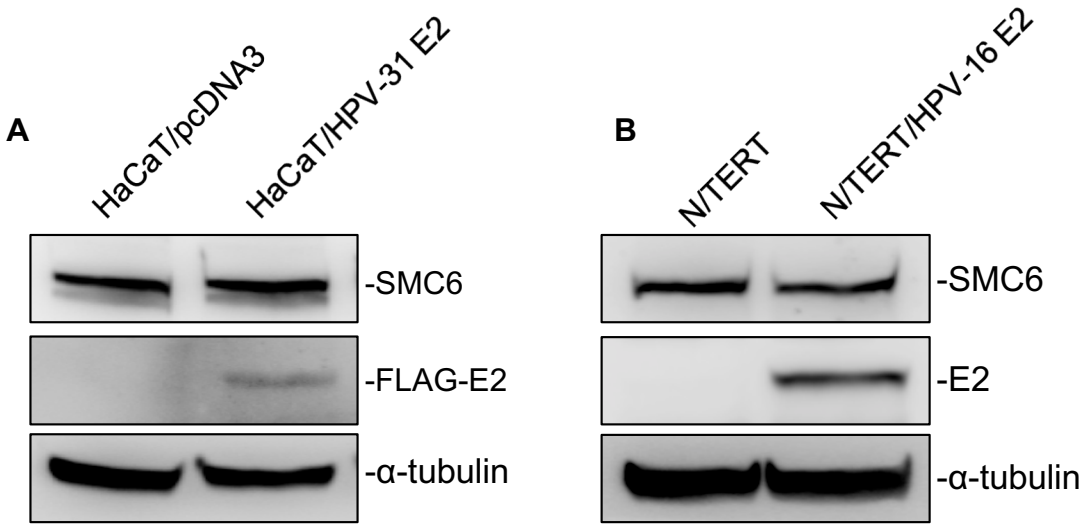

Figure 3.9. HPV-31/-16 E2 do not affect SMC6 levels. (**A**) Total protein was isolated from control and HPV-31 FLAG-E2 expressing HaCaT cells, then immunoblotting performed using mouse M2 anti-FLAG, rabbit anti-SMC6, and mouse DM1A anti-α-tubulin antibodies. (**B**) As in A, but with parental N/TERT and N/TERT cells expressing HPV-16 E2 and immunoblotting with mouse TVG-261 anti-HPV-16 E2 antibody for E2 detection.

3.6 Episomal versus Integrated HPV-31 Effects on SMC6 Protein Levels

HPV E2 regulates the transcription of viral oncoproteins and loss of E2 function following genome derepresses HPV E6 and E7 expression [116-118]. While I observed no difference in SMC6 stability in response to HPV-31/-16 E2 expression alone, I questioned whether integration and subsequent derepression of viral genome transcription may alter SMC6 levels in the context of viral infection. I isolated total protein from parental, episomal HPV-31 maintaining CIN612-9E cells and CIN612-9Ei cells, which are derived from CIN612-9E cells but possess integrated viral genomes, then compared total SMC6 protein levels (Figure 3.10). Cells maintaining episomal HPV-31 had reduced SMC6 protein expression compared to CIN612-9E cells with integrated HPV-31. Densitometry revealed that there was a statistically significant increase in SMC6 levels of nearly 2-fold in CIN612-9Ei cells as compared to parental CIN612-9E cells. These data suggest that integration of viral genomes increases SMC6 abundance, suggesting that other viral factors may influence SMC6 stability when E2 regulatory functions are lost.

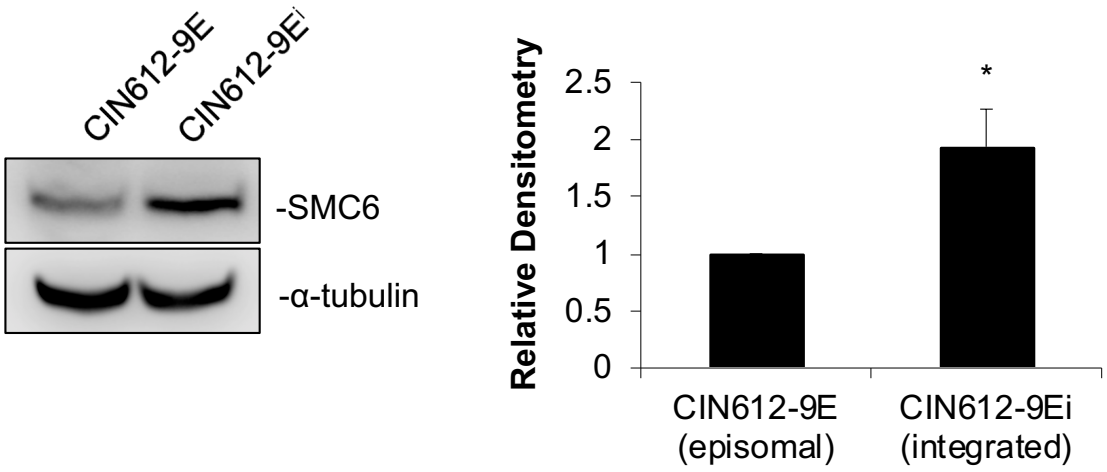

Figure 3.10. SMC6 expression is greater in integrated HPV-31 CIN612-9E compared to parental, episomal HPV-31 CIN612-9E cells. (**Left**) Total protein was isolated from CIN612-9E (episomal HPV-31) and CIN612-9Ei (integrated HPV-31) cells and immunoblotting for SMC6 and α-tubulin performed. (**Right**) Densitometry was calculated relative to α-tubulin and statistical significance determined by two-tailed Student's T-test. Experiments were performed three independent times and * = $\boldsymbol{p} < 0.05$.

CHAPTER 4

DISCUSSION

In this study, I investigated SMC6 interactions with HPV proteins and explored the role of SMC5/6 during the maintenance phase of the HPV-31 life cycle. I found SMC6 is associated with HPV-31 E2 by co-IP and have further identified that this interaction requires the E2 transactivation domain. While I cannot rule out a contribution of the E2 DNA-binding domain, these data infer that SMC6 interacts with full-length HPV-31 E2. Importantly, I demonstrated transient knockdown of SMC6 and NSE3 resulted in increased HPV-31 replication and transcription, implying that SMC5/6 represses these processes during the maintenance phase of the viral life cycle. I had speculated that SMC5/6-mediated repression of HPV replication may occur through interactions of SMC6 with E1/E2 complexes as a mechanism utilized by E2 to prevent over-replication of viral genomes. Data indicate this to be an unlikely explanation of the observed SMC5/6 repression of viral replication and transcription as I did not find an interaction of SMC6 or NSE3 subunits with either HPV-31 E1/E2 complexes or E1. However, data showed that the association of SMC6 with E2 was reduced in the presence of E1, indicating that SMC6 and E1 compete for E2 binding. This possibility is supported in that E2 interactions with both E1 [63-66] and SMC6 occur via the E2 TAD. Initiation of viral replication requires E2-mediated recruitment of E1 to the viral ori and by inhibiting E1 access to the E2 TAD SMC6 could repress viral replication. My observations that viral replication was

increased in cells following depletion of SMC6 and NSE3 provide further evidence of this, as decreased E1 competition for E2 binding would enable increased E1 recruitment to viral genomes for initiation of replication. Together, these data imply that SMC5/6 may repress HPV-31 replication by inhibiting the ability of E1 to interact with E2 and subsequently be recruited to viral genomes to initiate replication. Whether SMC6 inhibits E2 interactions with other factors to mediate repression of the viral replicative program is not currently known.

SMC5/6 associates with host chromatin to mediate homologous recombination DNA repair, rescue stalled replication forks [165], and topologically entrap extrachromosomal DNA [166]. Additionally, SMC5/6 associates with hepatitis B [163, 164] and herpes simplex-1 [164] viral episomes. The purported localization of SMC6 to viral episomes and its association with E2 led us to speculate that SMC6 may be present on episomal HPV-31 genomes. Indeed, I found SMC6 and E2 were present on the LCR of HPV-31 episomes at both the 5' upstream E2BS and E2BS located adjacent to the viral ori and early promoter, but not the flanking L1 region, indicating specificity for where SMC6 localizes on viral genomes. Precisely how SMC5/6 is localized to viral and host DNA remains unclear and I wanted to determine if the presence of SMC6 at the viral ori was specific, or due to SMC6 non-specifically associating with DNA replication origins. I looked for the association of SMC6 and E2 at a known host replication origin and found E2 but not SMC6 was present, suggesting that the presence of SMC6 at replication origins is selective. E2 is known to associate with host DNA

[81, 183, 184] and its consensus sequences are found throughout the host genome [183]. I believe that the presence of E2 at the host replication origin is likely due to E2 interacting with host factors that are localized to the GM-CSF replication origin. Together these data show that SMC6 preferentially associates with the LCR of HPV-31 episomes, implying that SMC5/6 repressor functions may occur at the viral genome. Mechanistically, repression of HPV-31 replication and transcription could involve SMC5/6 influencing the accessibility of viral DNA through steric hindrance, recruitment of histone modifying proteins, or via interactions with E2 at the viral genome. It is also possible that E2 recruits SMC5/6 to viral episomes to regulate viral transcription and replication, as this is an established function of E2 [98]. Future work should seek to determine how SMC5/6 is targeted to viral DNA and if its repressor functions require localization to HPV episomes.

SMC5/6 inhibits the life cycle of hepatitis B (HBV) by repressing transcription of the non-replicating, covalently closed circular DNA (cccHBV) intermediate form of HBV [163, 164, 168, 169]. My data support an antiviral function for SMC5/6 that extends to repression of the HPV-31 replicative program, as SMC6 and NSE3 depletion enhanced viral replication and transcription. However, as HPV episomes are actively transcribed and replicated throughout the viral life cycle determining the repressor function of SMC5/6 is less straightforward, as it could involve repressing transcription, replication, or both. It is possible that the observed increase in viral replication was be due to increased transcription of

HPV E1 and E2 following SMC6 and NSE3 knockdown, or vice versa. For example, SMC6 depletion resulted in a nearly equivalent increase of viral E2, E8^E2, E1 and E1^E4 transcripts and DNA content. It is conceivable that increased viral replication could be resultant from higher levels of viral gene transcription. While E8^E2 is regarded as a repressor of viral replication and transcription whether increased E8^E2 transcript levels correlated with increased E8^E2 protein expression is not known. Conversely, SMC5/6 may repress HPV replication but not transcription. Should SMC5/6 function as a repressor of HPV replication alone then the resulting increase in viral replication following SMC6 and NSE3 knockdown would generate more viral DNA available to be transcribed. Consequently, any observed increase in viral transcript abundance would not be resultant from increased gene transcription, but from more viral genomes being transcribed. This question could potentially be answered by investigating the effects of SMC6 and NSE3 knockdown on viral transcription using replication defective viral genomes. HPV replication is E1-dependent and genomes that do not produce E1 remain transcriptionally competent but unable to replicate [186]. I have made one such HPV-16 E1 mutant genome by insertion of a translation termination linker into the E1 ORF (Figure A.1) and future work will seek to determine if SMC5/6 influences transcription of non-replicating HPV-16 during infection.

This study described a role for SMC5/6 as a repressor of the HPV-31 replicative program. While in agreement with the findings of studies examining the role of

the SMC5/6 complex in hepatitis B and herpes simplex-1 viruses, my findings contrast with a previous report in which SMC5/6 was not found to repress HPV-31 replication or transcription, but was instead necessary for maintenance of viral episomes [173]. This discrepancy has several possible explanations. For example, my study primarily relied upon transient depletion of SMC5/6 subunits SMC6 and NSE3, as sustained depletion of SMC5/6 subunits has been found to be lethal in both non-cancerous and cancerous cells types, as well as result in spontaneous DNA damage and chromosomal abnormalities [187]. It is possible that all of these factors contributed to findings that utilized stable SMC6 depletion (near complete loss) to evaluate the functions of SMC6 in episomal maintaining HPV-31 cells. Indeed, my attempts to generate uninfected and infected cell lines that tolerated a significant reduction or knockout of NSE3 and SMC6 were unsuccessful. This agrees with reports describing a functional SMC5/6 complex as essential for cell viability. Cells maintaining episomal HPV-31 that tolerated a marginal reduction in NSE3 did not have increased viral DNA content and I attribute this to the reasons described above. I speculate that accurately examining the effects of SMC5/6 depletion for determining its repressor functions during the HPV life cycle may be limited to transient assays.

The absence of NSE3 in complex with E2 suggests that E2 may alter SMC5/6 complex formation by excluding NSE3. SMC5 and SMC6 form heterodimers through binding of their central hinge region while closing of the SMC5/6 complex into its characteristic ring shape occurs by bridging of SMC5 and SMC6 terminal

globular domains by kleisin subunit NSE4 [147]. Both NSE1 and NSE3 are joined to the SMC5/6 complex by binding NSE4. Should E2 interactions with SMC6 prevent NSE4 binding, the NSE3 association with the SMC5/6 complex would be lost. While this possibility accounts for the observed absence of NSE3 I believe it to be unlikely. NSE3 is required for SMC5/6 DNA binding [151] and stability of the SMC5/6 complex [153]. Therefore, exclusion of NSE3 would require that the SMC5/6 complex be stabilized and obtain DNA binding functionality. I do not believe that E2 disruption of SMC5/6 complex formation is a likely explanation for the absence of NSE3 during E2 IP for multiple reasons. For example, input samples from all IP experiments showed that NSE3 abundance was not altered in response to co-transfection of cells with E2. Furthermore, in experiments where SMC6 was pulled down the amount of NSE3 present in IP samples was unchanged when E2 was present. However, without internal loading controls I cannot exclude the possibility that low levels of NSE3 were degraded and E2 sequesters a fraction of SMC6 to form a separate complex that does not include NSE3.

HPV E2 proteins have been reported to influence gene expression and protein stability of host factors [79, 185], but I do not find that either HPV-31 or HPV-16 E2 proteins alter SMC6 stability in human keratinocytes. This finding agrees with those examining HPV-5, HPV-18, and BPV-1 in that E2 was not found to affect SMC6 abundance [173], but contrasts with studies of hepatitis B virus (HBV) wherein the master regulatory protein of HBV, HBx, was found to target SMC5/6

for degradation shortly after infection [163, 168]. It is possible that this is due to differences in the cell type infected and differing roles of SMC5/6 in the life cycles of these viruses. While HBV infects non-dividing human hepatocytes, actively dividing non-cancerous and cancerous human cell lines have demonstrated that sustained loss of SMC5/6 is lethal [187]. The replicative program of HPV is dependent upon maintaining the actively dividing state of infected basal keratinocytes and therefore, while loss of SMC5/6 may be tolerable in non-dividing HBV-infected cells, actively dividing HPV infected keratinocytes would require a functional SMC5/6 complex for completion of the HPV life cycle.

Loss of E2 function leads to unregulated viral genome transcription and results in the derepression of HPV E6 and E7 expression [116-118]. Cells maintaining integrated HPV-31 genomes had elevated levels of SMC6 protein as compared to parental, episomal HPV-31 maintaining cells and this is potentially explained in that the presumed loss of E2-mediated regulation of transcription would result in increased E7 expression. E7 is known to stimulate the DNA damage pathway [182] and this could potentially cause increased expression of SMC6. Alternatively, altered expression of other viral factors may influence SMC6 expression or stability. However, future studies are needed to determine how viral genome integration contributes to increased SMC6 expression.

In conclusion, this study has identified a novel role for SMC5/6 in the life cycle of HPV-31. I have demonstrated that depletion of SMC6 and NSE3 result in

enhanced viral replication and transcription, inferring that SMC5/6 is a repressor of the HPV-31 replicative program. While the repressor function of SMC5/6 in hepatitis B virus and herpes simplex-1 viral infections appears to be repression of viral DNA transcription, I have not determined if the antiviral functions of SMC5/6 during HPV infection are specific to replication, transcription, or both. I have demonstrated that SMC6 is present on episomal HPV-31 genomes and interacts with HPV E2 proteins, but the mechanism by which SMC5/6 represses the viral replicative program remains unclear. I hypothesize that SMC5/6-mediated repression may involve inhibiting initiation of viral replication through competition with E1 for E2 binding. Taken together, this study has demonstrated that the antiviral roles of SMC5/6 include functioning as a repressor of the HPV-31 replicative program.

CHAPTER 5

ADDITIONAL EXPERIMENTS

5.1 Development of a Novel and Efficient Method of Gene Transfer

5.5.1 Introduction

Human papillomaviruses are double-stranded DNA viruses that infect basal keratinocytes of the skin and mucosal epithelium [2]. The viral genome encodes 8 proteins essential to the viral life-cycle, which are designated as early (E), or late (L), based upon their temporal expression during the HPV replicative program [32]. Among the late proteins are the viral major (L1) and minor (L2) capsid proteins that encapsidate the HPV genome. L1/L2 capsids produced *in vitro* have been found to efficiently package HPV genomes (quasivirions; QsV) and non-viral DNA (pseudovirions; PsV) not larger than 8 kilobases in size [188-191]. The ability of HPV capsids to package non-viral DNA represents a unique method for transferring plasmid DNA into cells and could provide an efficient and advantageous alternative to transfection-based methods.

L1 proteins are capable of spontaneous self-assembly into viral-like particles (VLPs) roughly 60nm in diameter that are nearly indistinguishable from native virions [20-24] (Figure 5.1). While L2 alone is not capable of forming VLPs its co-assembly with L1 improves the efficiency of VLP assembly [127]. Initial contact of

infectious virions with host cells involves L1 interactions with heparin sulfate proteoglycans (HSPGs) in the extracellular matrix and on the cell surface [122]. This initial interaction is believed to be essential for infection, as it drives conformational changes in the viral capsid that expose L2 to furin proteolytic cleavage necessary for capsid maturation [192]. Although L2 is not required for viral entry it is essential for proper genome trafficking within host cells, as DNA packaged by L1 alone fails to escape late endosomes [193]. L2 delivers viral genomes to the perinuclear space of infected cells via retrograde trafficking by the host cell retromer complex, where viral genomes will reside in trafficking vesicles until breakdown of the nuclear envelope during mitosis [123, 124]. Following breakdown of the nuclear envelope, L2 localizes viral genomes to PML nuclear bodies, where viral transcription and replication will take place [127].

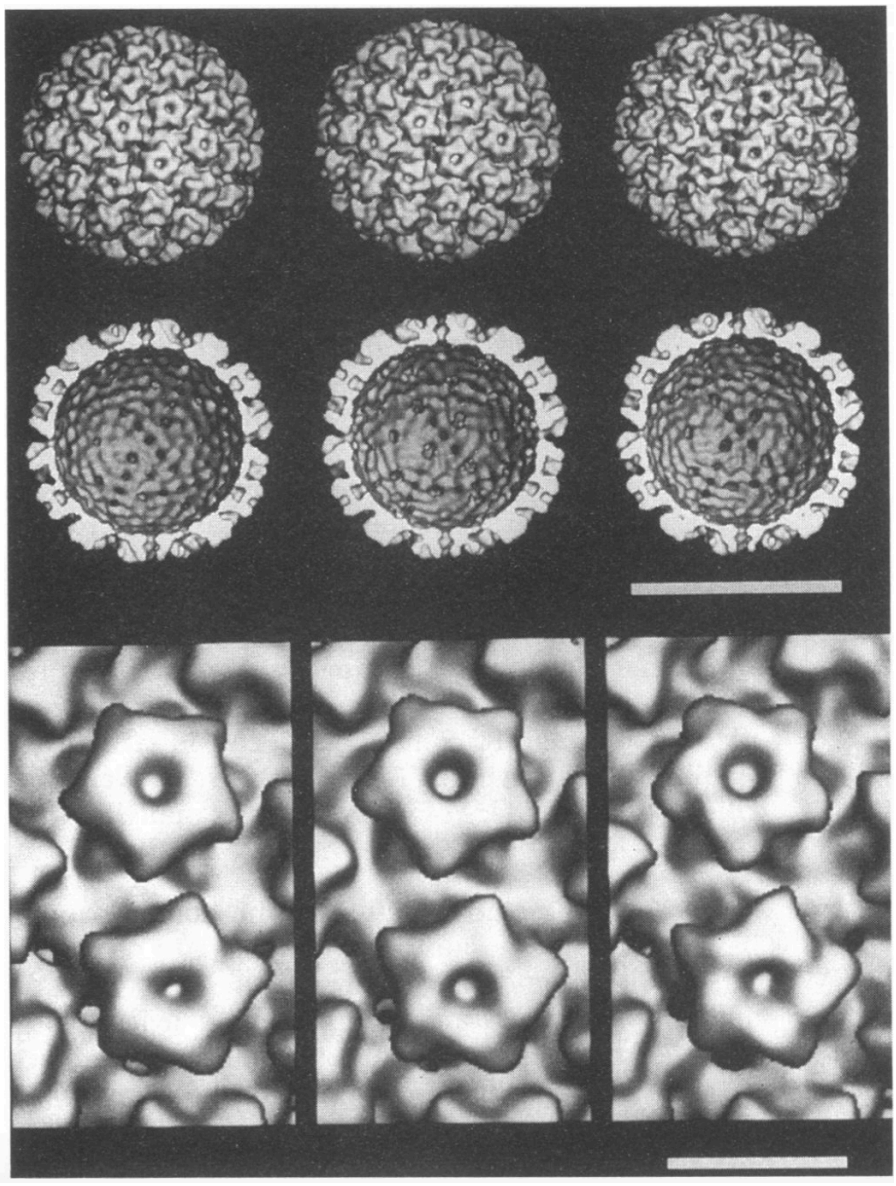

Figure 5.1. Surface-shaded Cryo-EM images of native HPV virions and viral-like particles. HPV-1 virion isolated from warts (left column), viral-like particle (VLP) comprised of L1 alone (middle column) and L1/L2 (right column). Exterior (top) and interior (middle) capsid view (bar = 50nm). L1 pentamer (bottom) close-up view (bar = 10nm). This figure was taken from [24].

Utilizing HPV capsids for gene transfer requires the ability to efficiently produce high titers of infectious virions. Initial studies of HPV infection were limited by inefficient and labor-intensive methods used for isolating HPV virions. Among such methods were isolation of virions from patient tumors or rafted keratinocytes, both of which resulted in low yields of virions obtained [194]. The discovery that co-transfection of plasmids encoding HPV L1 and L2 capsid proteins with DNA of interest (provided it be under 8 kilobases in size) provided a new and robust method that was less labor intensive and capable of generating high titers of infectious HPV virions [188-190, 195]. This method was further refined with the discovery that HPV virions produced in cells overexpressing the protease furin were significantly more infectious due to enhanced capsid maturation [192, 196]. Despite the development of new and robust methods of virion production the efficiency of different cell lines to be infected remained variable. This has led to attempts to develop more efficient methods in facilitating infection with HPV PsV, such as pre-binding virions to a keratinocyte generated extracellular matrix (ECM) prior to infection of target cells [197, 198].

In this study I sought to develop a more efficient method for gene delivery in cells that are difficult to transfect and infect using HPV PsV techniques. I describe a new HPV L1/L2-based PsV infection method termed suspension-mediated infection (SMI) and evaluated the efficiency of SMI in comparison to other currently used methods in multiple cell types. SMI was shown to efficiently mediates gene transfer in difficult to transfect cell lines, as well as in those cell

lines in which other PsV methods fail to produce significant infection. Together, I find that SMI is an adaptable and efficient method for facilitating infection of different cell types with PsV.

5.5.2 Materials & Methods

Cell Culture

C33A, HaCaT, HeLa, J2 3T3, HepG2, Huh-7, HEK293TT, HEK293TTF, and SH-SY5Y were cultured in DMEM/10% FBS. CIN612-9E cells were maintained in E-media with mitomycin-C treated J2 cells. N/TERT cells were maintained in KSFM supplemented with human EGF and bovine pituitary extract. Spontaneously immortalized keratinocyte (NIKS) were cultured in F-media with mitomycin-C treated J2 cells.

PsV Production

PsV were produced by packaging pmCherry, HPV-31 V5-E2, HA-εCOP, or cccHBV(+) (Figure 5.2) in HPV-16 L1/L2 capsids according to previously described methods [196, 199]. Briefly, 20 µg p16sheLL (Figure 5.3) was co-transfected into HEK293TTF cells with either pmCherry, HPV-31 V5-E2, or HA-εCOP using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and cells incubated overnight. The use of p16sheLL avoids undesirable self-packaging of the L1/L2 construct. The following day media was changed, non-essential amino acids (Gibco) added and cells returned to incubate for an additional 24 hours. Two-days post-transfection cells were collected and washed in 1X PBS, then 1.5X cell pellet volumes 1X PBS/9.5mM $MgCl_2$ added and cells

gently resuspended (e.g. 100µL cell pellet observed in comparison to fluid dummy would have 150µL 1X PBS/9.5 mM $MgCl_2$ added, then for all subsequent calculations the cell pellet volume (CPV) would be considered as 250 µL). Next, maturation buffer was added as follows:

- 1/20th CPV 10% Triton X-100 (C_f = 0.5%)
- 1/40th CPV 1M $(NH_4)_2SO_4$ (pH 9; C_f = 25mM)
- 1/10th CPV 50mM $CaCl_2$ (C_f = 5mM)

Cells were then gently resuspended and left to incubate at 37^{o}C for 48 hours in siliconized microcentrifuge tubes. Next, lysates were chilled on ice for 5min, followed by centrifugation at 4^{o}C for 5min at 5,000 x *g* and then PsV containing supernatant transferred to fresh siliconized tubes. Original cell pellets were then resuspended in 1X cell pellet volumes 1X PBS, then spun again at 4^{o}C and combined with the previous supernatant. Combined supernatants were re-clarified by repeating the centrifugation, then supernatant containing PsV transferred to fresh tubes and stored at 4^{o}C. All infection methods tested (pre-plated, ECM, SMI) when determining efficiency of infection were performed a minimum of three independent times using 3µL mCherry PsV and 1 x10^4 cells.

cccHBV(+) Digestion and Re-ligation

EcoRI digestion of cccHBV(+)/pSP was performed at 37^{o}C to separate cccHBV(+) and pSP backbone. EcoRI was then heat inactivated by incubating samples at 65^{o}C for 20min. cccHBV(+) episomes were ligated overnight at room temperature under dilute conditions to minimize concatemer formation, then purified according to previously described methods [200].

Encapsidated DNA Analysis

PsV packaging of cccHBV(+) was evaluated by qPCR and nuclease resistance. Samples were undigested (unencapsidated DNA), Proteinase K only (total cccHBV(+) DNA in PsV prep), Benzonase only (control for nuclease activity), and sequential digestion with Benzonase then Proteinase K (encapsidated DNA). Benzonase digestion was performed at 37^{o}C for 30min. Proteinase K only digestion was performed at 50^{o}C for 30min, then Proteinase K inactivated using PMSF. Double digest was performed by first digesting with Benzonase, followed by Proteinase K digestion and inactivation as described above. qPCR C_t values were used to calculate fold change of packaged viral DNA to unpackaged (set to one).

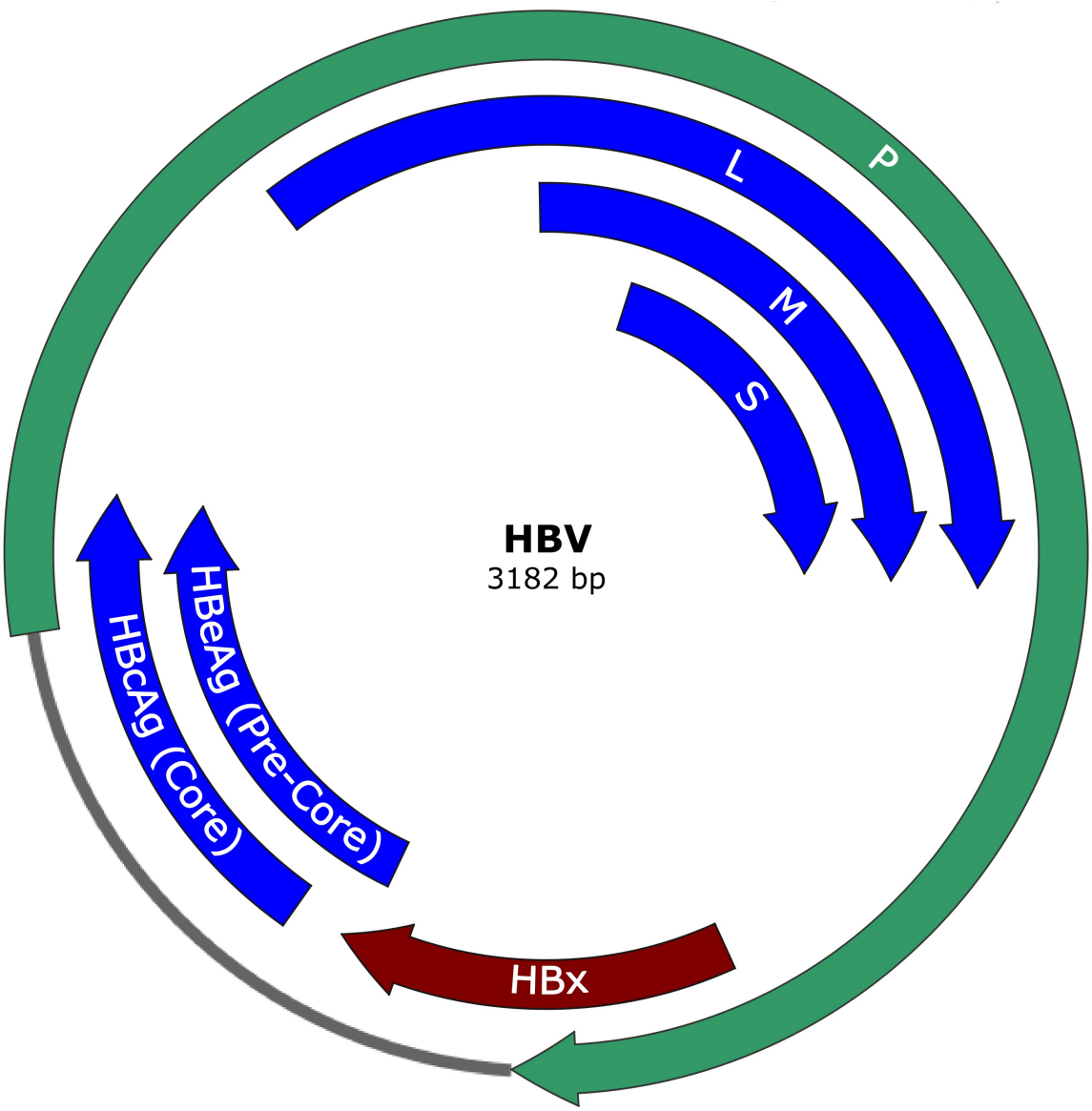

Figure 5.2. HBV cccDNA. The 3.2kb HBV cccDNA episome functions as the transcriptional template for production of viral mRNA encoding the polymerase (P), HBx transcription factor (red), pre-core (HBeAg), core (HBcAg), large (L), medium (M), and small (S) surface antigens.

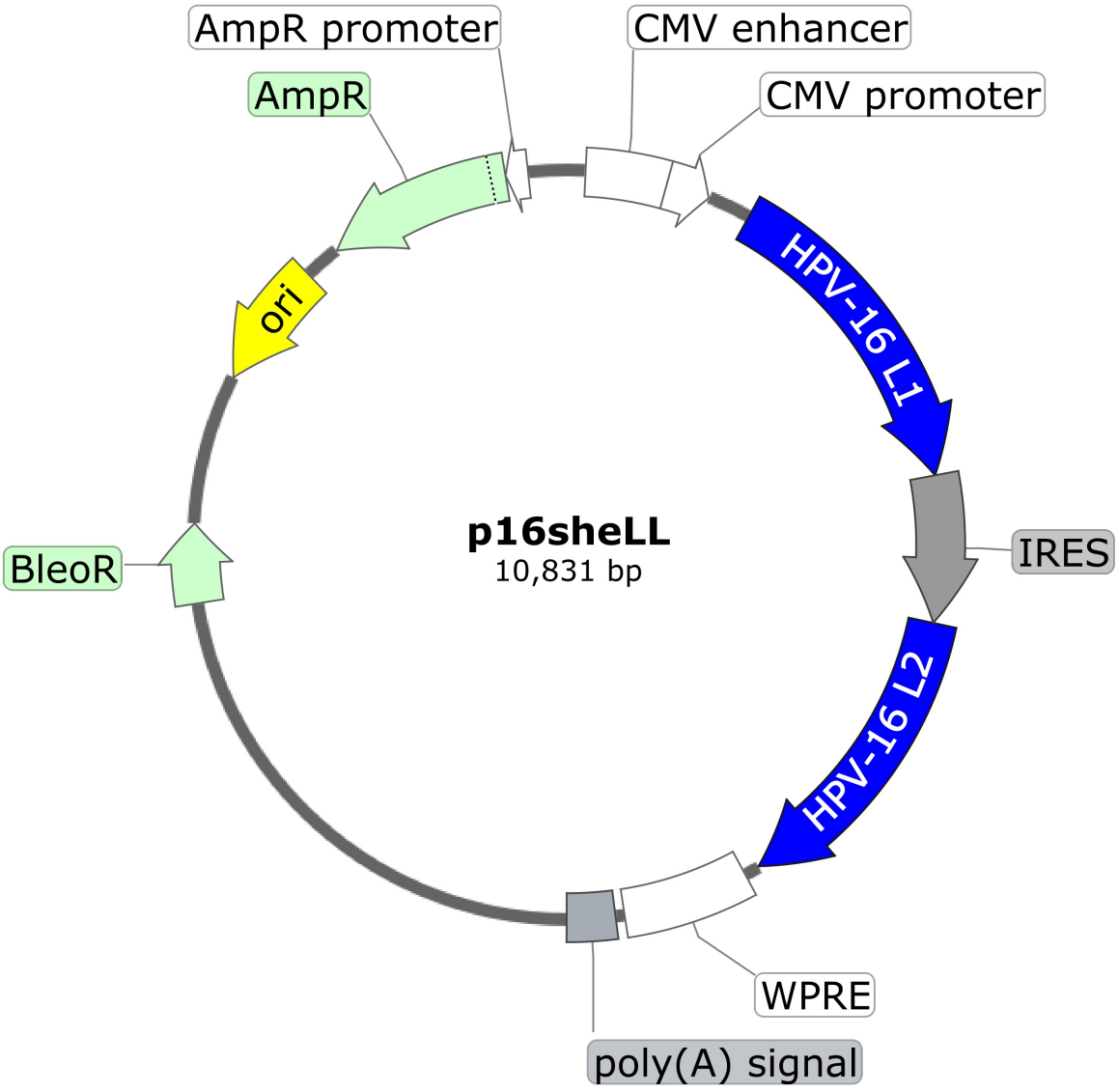

Figure 5.3. p16sheLL. The HPV-16 p16sheLL vector encodes both major (L1) and minor (L2) capsid proteins used to package viral and non-viral DNA. This plasmid was provided by John Schiller and previously described in [201].

Pre-plated Infection

Cells were plated without pre-generated ECM prior to infection, allowed to fully adhere to plates and then PsV added. Twenty-four hours later media was replaced and fluorescence observed at twenty-four, forty-eight, and seventy-two hours post-infection. Efficiency of infection was determined by the percent of intracellular red fluorescence.

Extracellular Matrix (ECM) Generation & Infection

ECM was produced according to previously described methods [198]. To generate ECM cells were grown to confluence, then washed with 1X PBS and treated with ECM-buffer (170 mM NH_4OH, 0.5% Triton X-100, 1X PBS). Following visual confirmation that cell bodies had been removed using light microscopy, ECM buffer was removed and then gently washed three times with 1X PBS. PsV and media (containing FBS) were added to wells and PsV allowed to adhere to ECM overnight at 37^{o}C. The following day, media and unbound PsV were removed and ECM gently washed with 1X PBS, then standard culture media and cells added. Infection efficiency was determined as described above.

Suspension-mediated Infection (SMI)

SMI was performed by mixing cells and PsV in suspension at the time of plating, allowing PsV to bind to cells in suspension prior to adhesion to plates and in the absence of ECM, then cells were incubated overnight at 37^{o}C (Figure 5.4). The following day, media containing unbound virus was removed and cells returned to standard culture conditions. Infection efficiency was determined as described above.

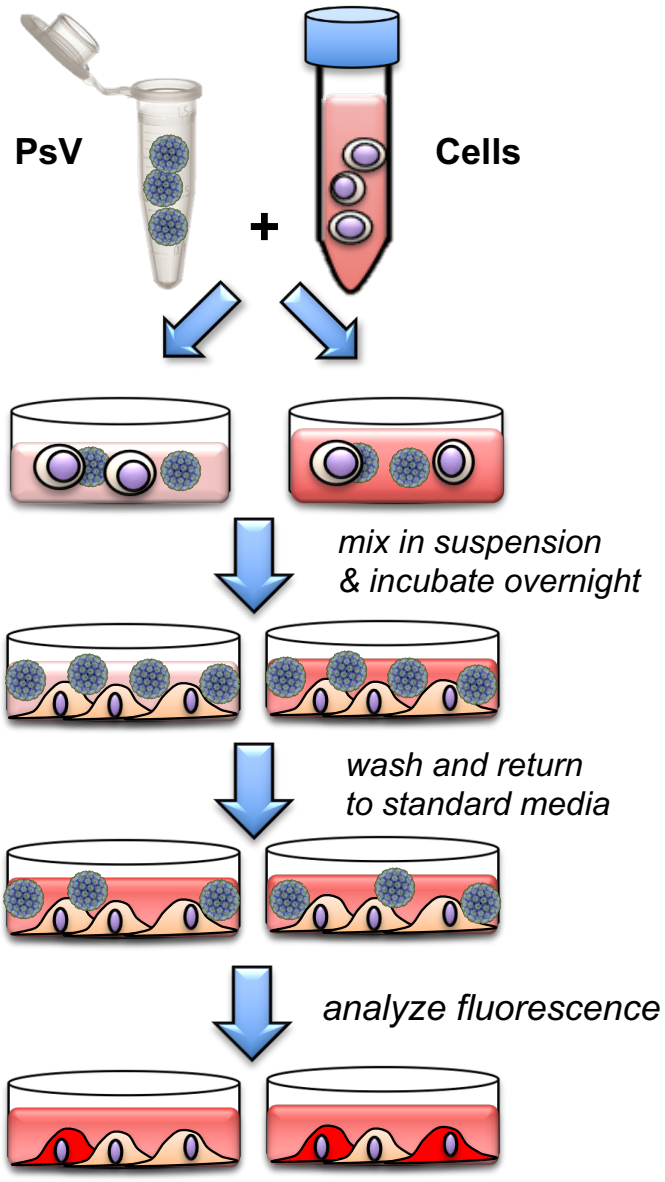

Figure 5.4. Suspension-mediated infection (SMI). Schematic representation of the suspension-mediated infection (SMI) method of PsV infection.

Immunoprecipitation & Immunoblotting

HEK293TT, N/TERT and SH-SY5Y cells were infected with mCherry, HPV-31 V5-E2, or HA-εCOP PsV. Forty-eight hours later, cells were lysed in 0.5% NP-40/150mM NaCl/50 mM Tris-HCl (pH 8)/10% glycerol and protease inhibitor cocktail (Sigma). Samples were then rotated for 1hr at 4^{o}C after the addition of 4μL benzonase (Millipore), then spun for 10min at 4^{o}C and supernatant collected. IP was performed by incubation of lysates with Protein A/G slurry and either rabbit anti-V5 (Cell Signal Technologies) or mouse 12CA5A1 anti-HA antibodies. Beads were then washed in lysis buffer and boiled in 2X Laemmli buffer. SDS-PAGE and subsequent Western blot were then performed as described previously.

5.5.3 Results

Immortalized non-cancerous keratinocyte cell lines such as N/TERT and HaCaT are commonly used for examination of the HPV life cycle. We first sought to determine efficiency and optimize conditions for infection of these cell lines. PsV packaged with a plasmid encoding mCherry red protein were used to calculate infection efficiency as the percentage of red fluorescent cells, which indicated nuclear uptake and gene expression of plasmids. We first titrated PsV by infecting HEK293TT cells with 1-10μL of virus to identify the volume of PsV that

resulted in roughly 90-95% mCherry positive cells. Two days post infection we observed 100% infection with 5μL and 10μL volumes, which indicated PsV were in excess (Figure 5.5A), while 3μL resulted in 90-95% mCherry positive cells. All subsequent infections used 3μL mCherry PsV. Several independently produced mCherry PsV batches demonstrated consistent infectivity, indicating equivalent titers are produced between batches. Infection efficiency of N/TERT and HaCaT cells were significantly reduced compared to HEK293TT and HeLa cells (Figure 5.5B). Low infectivity of N/TERT cells is consistent with previously published results [202].

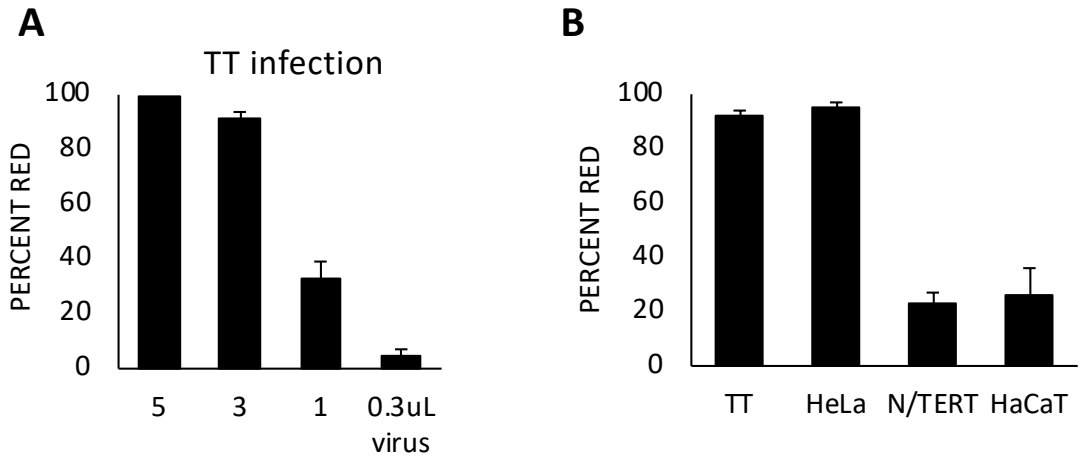

Figure 5.5. mCherry PsV titration and infection. (A) mCherry PsV was titered by adding the indicated volumes of virus to HEK293TT cells and evaluating intracellular red fluorescence 48 hours later. (**B**) Indicated cell lines were cultured overnight before infection with 3μL mCherry PsV and intracellular red fluorescence examined 48 hours later. All experiments were performed at least 3 independent times and values shown as mean ± SEM.

During infection HPV binds to factors in the cellular ECM prior to interacting with cell surface receptors, which is believed to induce conformational changes in the capsid that promote internalization [122]. HPV has been found to bind epithelial cell derived ECM with the greatest efficiency [197, 198] and some labs have used ECM to study HPV infection. We questioned whether infection efficiency of HaCaT and N/TERT cells could be increased by pre-binding of PsV to keratinocyte ECM prior to infection. HaCaT, N/TERT and NIKS cells were grown to confluence and ECM generated by removing cells using NH_4OH as described elsewhere [198]. mCherry PsV were allowed to adhere to the ECM overnight and the following day cells were added. Keratinocyte ECM promoted increased infectivity in both HaCaT and N/TERT cells as compared to infections performed without ECM (Figure 5.6). Efficient infection of N/TERT cells using ECM requires that FBS is included in the media during incubation of PsV with the ECM. To determine if ECM could be used to promote infection of non-keratinocyte cell lines I tested whether HepG2, Huh-7, or N/TERT ECM could be used to promote infection of HepG2 and Huh-7 cells. Roughly 100% of control HEK293TT cells were infected without ECM while no infection was observed in HepG2 cells with or without ECM (Figure 5.7). Huh-7 cells demonstrated low infectivity without ECM or with Huh-7 ECM, but nearly 80% infection was observed when Huh-7 cells were infected using N/TERT ECM.

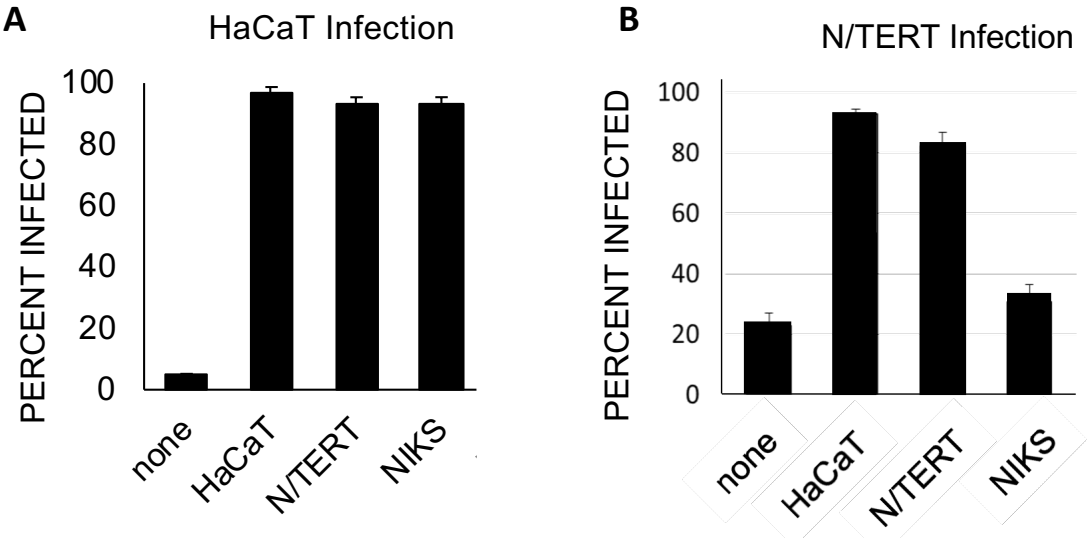

Figure 5.6. Keratinocyte ECM enhances HaCaT and N/TERT infection. ECMs from indicated cells at the bottom of each graph were incubated with mCherry PsV overnight in FBS containing media. Unbound PsV were removed, fresh media and HaCaT **(A)** or N/TERT **(B)** cells added to ECM. Cells infected without ECM (none) were plated the previous day and infections were performed at the same time. Infection efficiency was determined by the percent of intracellular red fluorescence. All experiments were performed at least 3 independent times and values shown are as mean ± SEM.

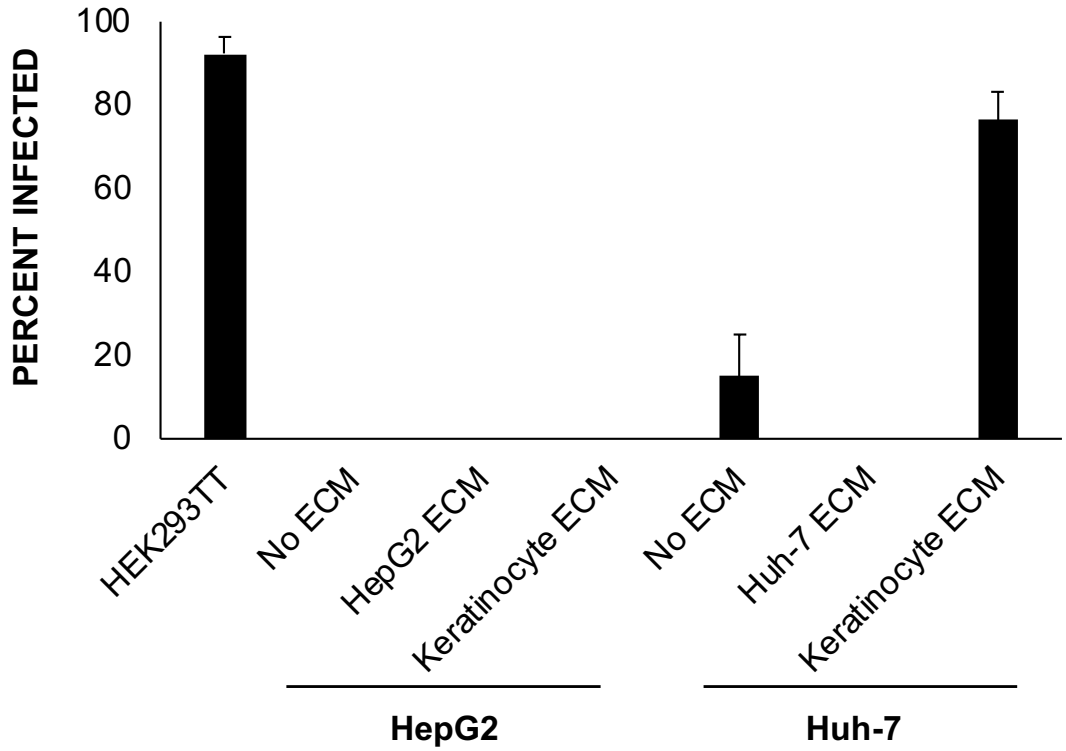

Figure 5.7. mCherry PsV hepatocyte infections. ECM from HepG2, Huh-7 or N/TERT cells were incubated with mCherry PsV overnight. Unbound PsV were removed, ECM washed and cells added to ECM. Control HEK293TT, HepG2 and Huh-7 cells infected without ECM (No ECM) were plated the previous day and infections performed at the same time as ECM infections. Infection efficiency was determined by the percent of intracellular red fluorescence 72hrs post-infection. All experiments were performed at least 3 independent times and values shown are as mean ± SEM.

The established model of HPV infection involves interactions of viral capsid proteins with cellular factors in the ECM and on the cell surface to mediate internalization of the virus [122]. I speculated that infecting cells in suspension may enhance infection by enabling increased PsV access to a larger cell surface area and more viral attachment points for stimulating internalization. I piloted trials of suspension-mediated infection (SMI) in which trypsinized cells are exposed to PsV immediately before plating (Figure 5.4). SMI infection efficiency was increased or equivalent to cells plated prior to infection (Figure 5.8). N/TERT and Huh-7 cells demonstrate low infection efficiency without ECM but were efficiently infected with PsV using SMI. HepG2 cells were uninfectable, even with ECM by PsV but roughly 100% susceptible to infection by SMI. Representative images of SMI infected cells are shown in Figure 5.9 with differential interference contrast white light images on left and mCherry PsV infected cells on right. Our lab has previously observed that incubating various ECM in different media can influence infection efficiency. I speculated that this might also enhance infection efficiency when using SMI. I found that SMI efficiency in various cell lines could be significantly increased based on the use of different media (Table A.7). For example, N/TERT cells infected in KSFM with mCherry PsV showed marginal infection efficiency using SMI, but when SMI was used on N/TERT cells in F-media, efficiency increased from approximately 10% to roughly 80% (Figure 5.10). N/TERT cells are able to proliferate in serum containing media and this induces morphological change, however cells begin reverting back within roughly 30 minutes of placement into serum-free KSFM (Figure 5.11). SMI efficiency in

HepG2 and Huh-7 cells is also influenced by media, with E-media producing the highest infection efficiency in both cell lines (Figure 5.10).

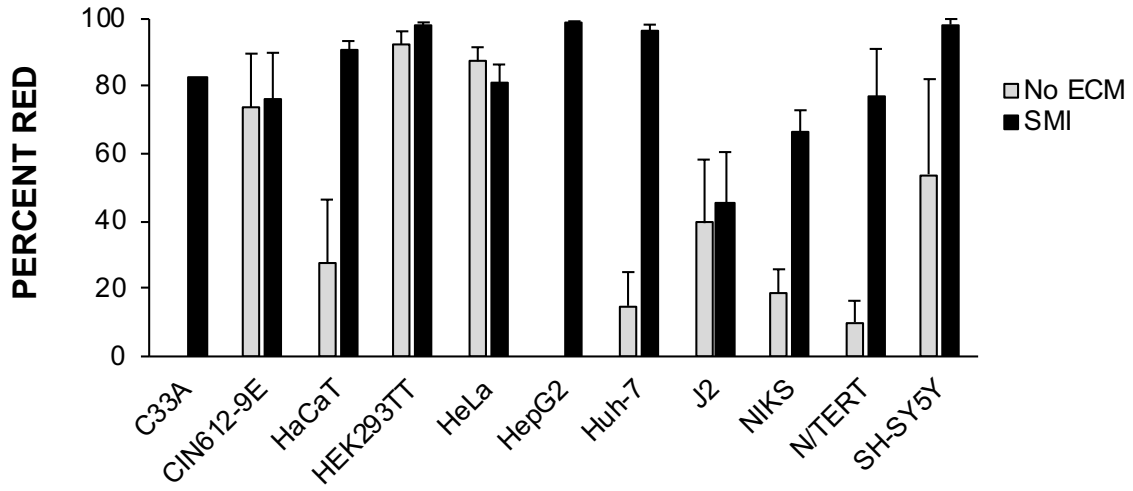

Figure 5.8. SMI efficiently infects diverse cell types. Summary comparison of cell infection efficiency at 72hrs post-infection with mCherry PsV using media found most efficient for infecting each cell type. Cells were plated without ECM prior to infection (grey), or infected by SMI (black). Data are percent of cells positive for mCherry fluorescence. All experiments were performed at least 3 independent times and values are expressed as the mean of +/- SEM.

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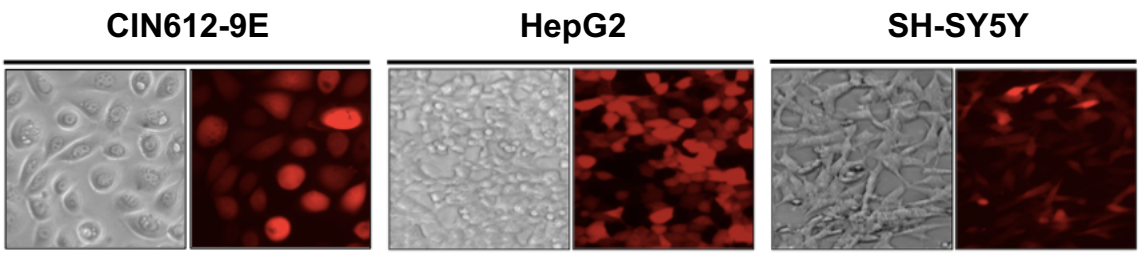

Figure 5.9. SMI infection immunofluorescence. Immunofluorescence of CIN612-9E, HepG2 and SH-SY5Y cells 72 hours after infection by SMI with mCherry PsV. DIC image on left and fluorescent mCherry images on right.

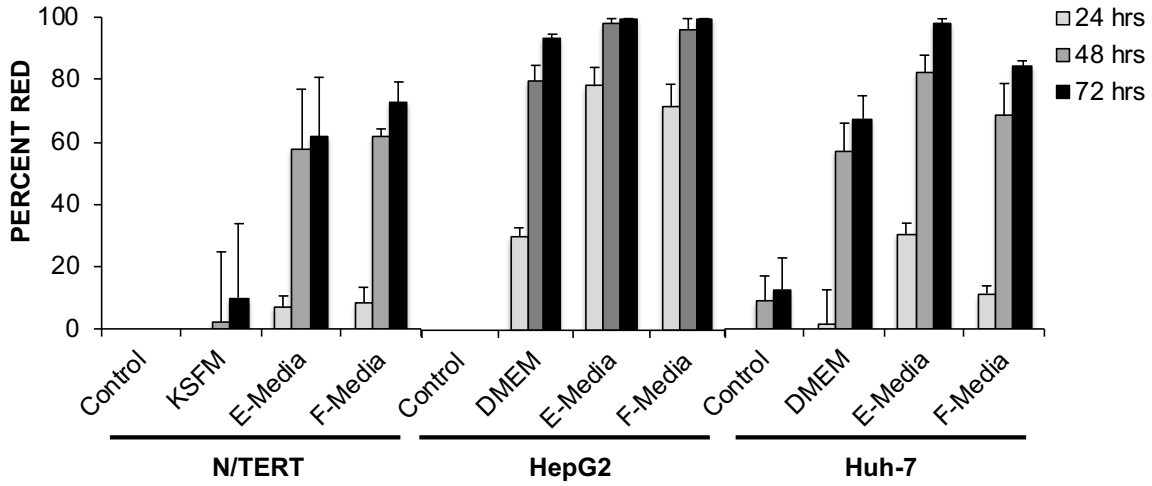

Figure 5.10. Media influences SMI efficiency. N/TERT, HepG2, and Huh-7 cells were infected with mCherry PsV without ECM (control), or using SMI in different media and fluorescence analyzed at 24, 48, and 72hrs. All experiments were performed at least 3 independent times and values are expressed as the mean +/- SEM.

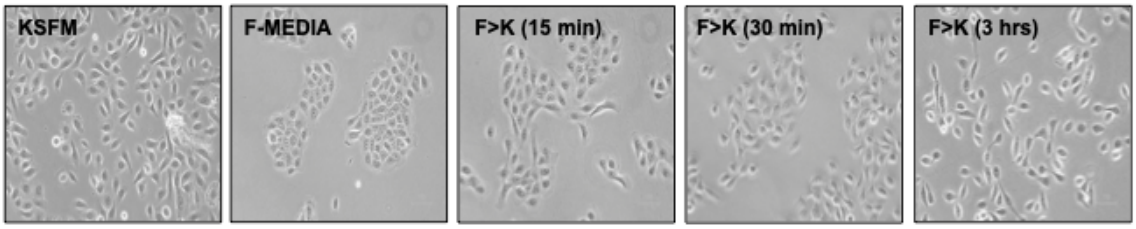

Figure 5.11. FBS influences N/TERT cell morphology. Morphology of N/TERT cells observed after plating cells into either KSFM (panel 1) or F-media containing FBS (panel 2), then N/TERT cells 15min after changing F-media to KSFM (panel 3), 30min (panel 4), and 3hrs (panel 5).

Given that efficient infection of difficult to transfect cell lines (e.g. N/TERT, SH-SY5Y) was demonstrated and PsV capsids can package plasmid DNA up to 8 kilobases in size, I sought to determine if PsV infection using SMI could be used as an alternative to transfection with delivering different plasmids. Using SMI, HEK293TT, N/TERT and SH-SY5Y cells were infected with either mCherry, HPV-31 V5-E2, or HA-εCOP PsV. Two days post-infection, cells were lysed and immunoprecipitated with antibody to V5 or HA. Successful infection and plasmid expression was observed in all cell lines (Figure 5.12A-D). Therefore, SMI constitutes an employable method to mediate efficient plasmid delivery and an alternative to transfection-based methods. SMI and ECM based methods both efficiently infect N/TERT cells and these methods were compared for heterologous protein expression. N/TERT cells were infected with V5-E2 PsV either by SMI or ECM method, then two days post-infection lysates were immunoblotted with V5 antibody. Intriguingly, SMI produced greater V5-E2 protein expression as compared to ECM (Figure 5.12D).

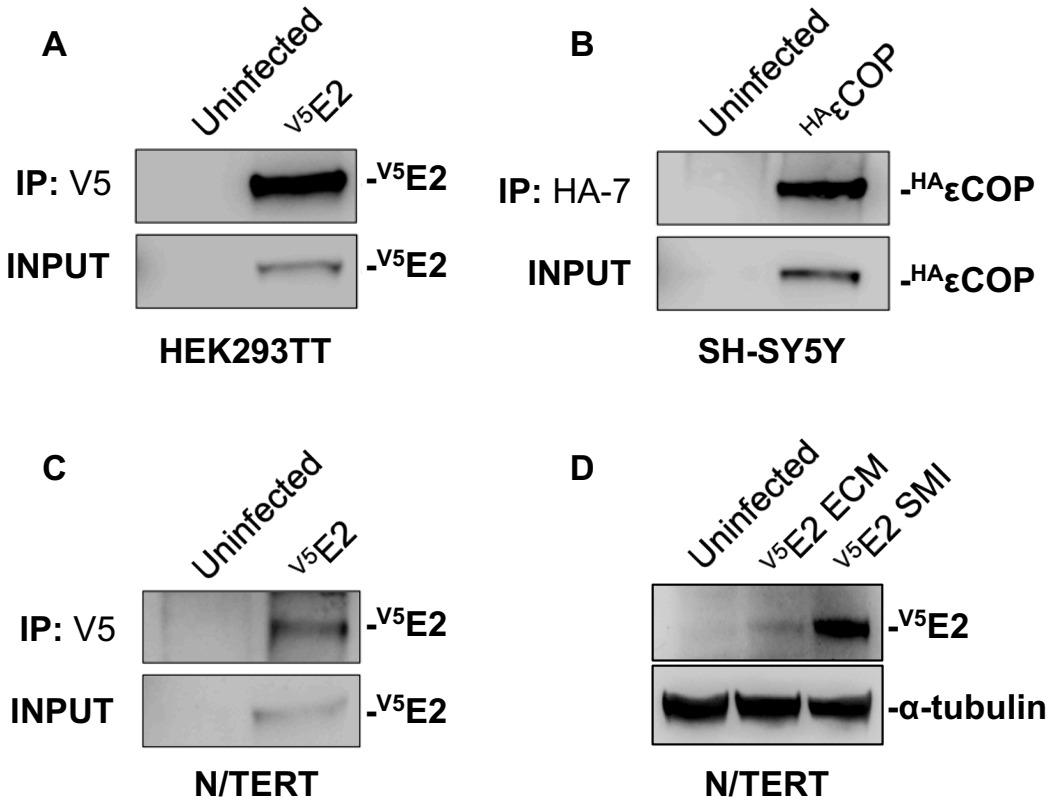

Figure 5.12. SMI efficiently delivers genes for protein expression. (**A**) HEK293TT cells were infected using SMI with HPV-31 V5-E2 PsV for two days, lysed and immunoprecipitated with rabbit anti-V5 antibody, then immunoblotted with mouse anti-V5 antibody. (**B**) SH-SY5Y cells were infected with HA-εCOP PsV for two days, lysed, and immunoprecipitated with mouse 12CA5A1 anti-HA antibody and immunoblotted with mouse HA-7 anti-HA. (**C**) As in A, but with N/TERT cells. (**D**) N/TERT cells were infected with V5-E2 PsV either by SMI or ECM methods. Two days following infection, cells were harvested and immunoblotted with V5 antibody.

Having demonstrated PsV can efficiently package different plasmids and infect HepG2 and Huh-7 hepatocytes, I wanted to determine if PsV could be used to package non-HPV viral DNA. Hepatitis B virus (HBV) utilizes a non-replicating, 3.2kb episomal form of the viral genome termed covalently closed circular DNA (cccDNA) to serve as a transcriptional template during infection [203]. I questioned if cccDNA could be packaged into PsV to provide an efficient method for directly delivering cccDNA into the nuclei of hepatocytes for studying HBV infection. I examined whether PsV had packaged HBV cccDNA(+) DNA by digesting samples with Benzonase and compared this to undigested samples. I observed roughly 2-fold more cccDNA(+) was packaged (nuclease resistant) than not packaged (undigested)(Figure 5.13). Benzonase only control digestion resulted in no signal amplification by qPCR, indicating complete digestion of unprotected DNA. These data suggest HPV PsV are capable of packaging hepatitis B virus cccDNA.

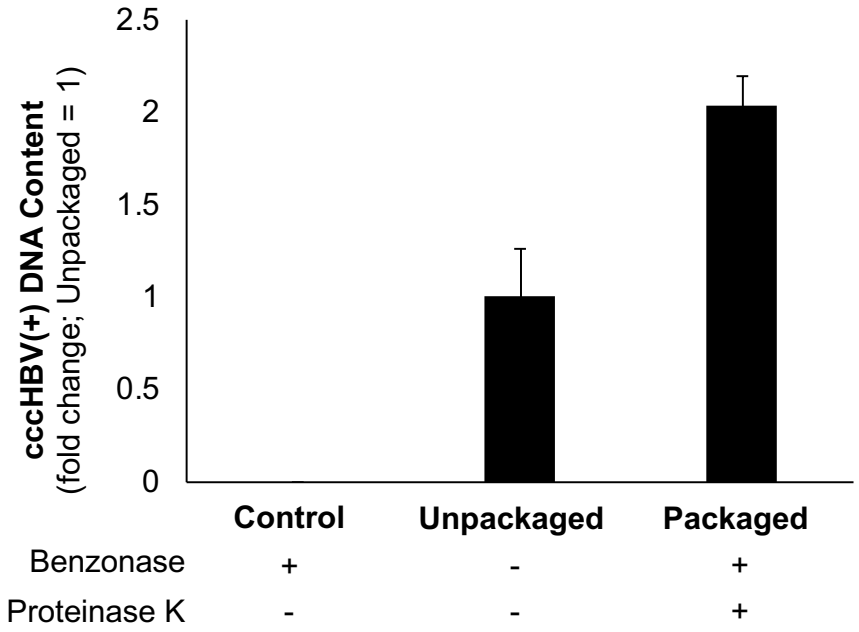

Figure 5.13. PsV package HBV cccDNA(+). Undigested PsV samples were used to determine unencapsidated (unpackaged) DNA. Nuclease digestion was performed to degrade unencapsidated DNA and Proteinase K digestion used to degrade Benzonase and liberate encapsidated (packaged) DNA. Benzonase only (control) was used to confirm nuclease activity. qPCR C_t values were normalized to total cccHBV(+) DNA present in PsV viral preps and fold change calculated as packaged over unpackaged (set to 1; n=1).

5.5.4 Discussion

The use of L1 and L2 proteins for production of infectious virions provides an efficient tool for packaging and delivering non-viral DNA by infection. However, while certain cell lines are readily infected by HPV capsids many others demonstrate low or variable infectability. mCherry packaged HPV pseudovirions (PsV) were used to evaluate the efficiency of different infection methods and describe a new method to mediate efficient gene transfer using PsV.

The ECM is comprised of different cellular factors that are involved in HPV capsid maturation [122]. Maturation of HPV capsids is believed to be driven by interactions with various cellular proteases of the ECM and heparin sulfate proteoglycans prior to internalization. I observed that keratinocyte ECM can be used to increase the infection efficiency of HaCaT and N/TERT cells provided that FBS is present. This is in agreement with others in that keratinocyte ECM can enhance HPV infection [197]. It is possible that the ECM method increases efficiency of infection in HaCaT and N/TERT cells by enhancing viral capsid maturation prior to cell contact. Additionally, the requirement for FBS to facilitate efficient N/TERT infection using ECM indicates that factors present in FBS enhance PsV maturation or binding with cells. While infection efficiency of some cell lines can be increased using keratinocyte ECM this is not always the case, such as with HepG2 cells. Why HepG2 cells are only infectable using SMI remains unclear, but there are several possible explanations. One possibility is

that HepG2 cells may produce factors that are inhibitory to infection which are proteolytically cleaved by trypsin when cells are suspended prior to infection by SMI. This possibility could be explored by removing HepG2 cells from plates using EDTA instead of trypsin, then infecting with mCherry PsV using SMI.

The requirement for HPV capsid interactions with the ECM for infection is challenged in the context of the SMI method. SMI mixes cells and PsV in suspension and results in efficient infection of multiple cell types, including those resistant to transfection (e.g. SH-SY5Y) or found uninfectable with or without ECM (e.g. HepG2). This is potentially explained by exposure of cells to PsV in suspension enabling increased cellular surface access or exposure to factors on the cell undersurface not normally accessible until mitosis as cells release from the culture plate. I believe that greater internalization of virions also provides a possible explanation as to why SMI was found more effective than ECM in delivering plasmids to overexpress protein. Why different media influenced infection efficiency of SMI may be explained by each media providing distinct factors that stimulate or inhibit infection. Alternatively, different media influencing the growth rates of cells could influence how quickly cells become infected. Viral entry into the nucleus requires breakdown of the nuclear envelope during cell division [124] and faster growing cells would enable more rapid access of the virus to the nucleus, with the opposite true for those with slowed growth rates. Multiple limitations are associated with transfection as a method for gene transfer and studying HPV biology. Transfected DNA is released into the cytoplasm and

detected by cGAS, resulting in cGAS-STING pathway activation [204, 205]. Conceivably, this could reduce plasmid expression at both transcriptional and translational levels. Variable transfection efficiency of different cell types makes reliable delivery and expression of plasmids challenging. Furthermore, the cost and toxicity of certain reagents highlights additional drawbacks of using transfection. These drawbacks are not limited to protein overexpression but also manifest when studying HPV biology. HPV genomes are not exposed to antiviral DNA sensors of the cytoplasm during natural infection and accurately studying HPV biology necessitates the avoidance of triggering pathways that would otherwise not be stimulated. Additionally, many keratinocytes used for studying HPV infection have low transfection efficiency. Together, the limitations associated with transfection-based approaches when studying HPV biology and for gene delivery highlight the need for alternative methods of gene transfer.

Transfection-based methods also preclude accurately studying certain aspects of hepatitis B virus (HBV) biology. HBV is trafficked to the nuclei of infected hepatocytes in the absence of cytoplasmic exposure and it is within the nucleus that HBV relaxed circular DNA is converted into cccDNA [203]. cccDNA is the persistent form of the virus that remains within infected hepatocytes and its elimination is necessary for curing chronic HBV infection, but current antiviral therapies do not target cccDNA [206]. Therefore, the ability to quickly and efficiently deliver cccDNA into the nucleus of cells could provide a novel approach for studying cccDNA in order to identify new therapeutic targets.

Further research is required to determine if PsV packaged cccDNA can be used to deliver transcriptionally active cccDNA into hepatocytes.

The ability of HPV capsids to package different plasmids could impart advantages to traditional transfection methods. I have shown that PsV are able to package both pCI and pcDNA3 based vectors. Furthermore, I have shown that HPV PsV are capable of packaging hepatitis B virus (HBV) cccDNA, which provides an intriguing new method for studying HBV infection. Additionally, I have shown efficient infection of difficult to transfect and infect cells using SMI. In conclusion, HPV PsV-mediated infections provide a more accurate method for studying HPV infection, as well as a versatile technique that can be used to efficiently deliver plasmids in different cell types and I have described a novel method for mediating this transfer.

APPENDIX

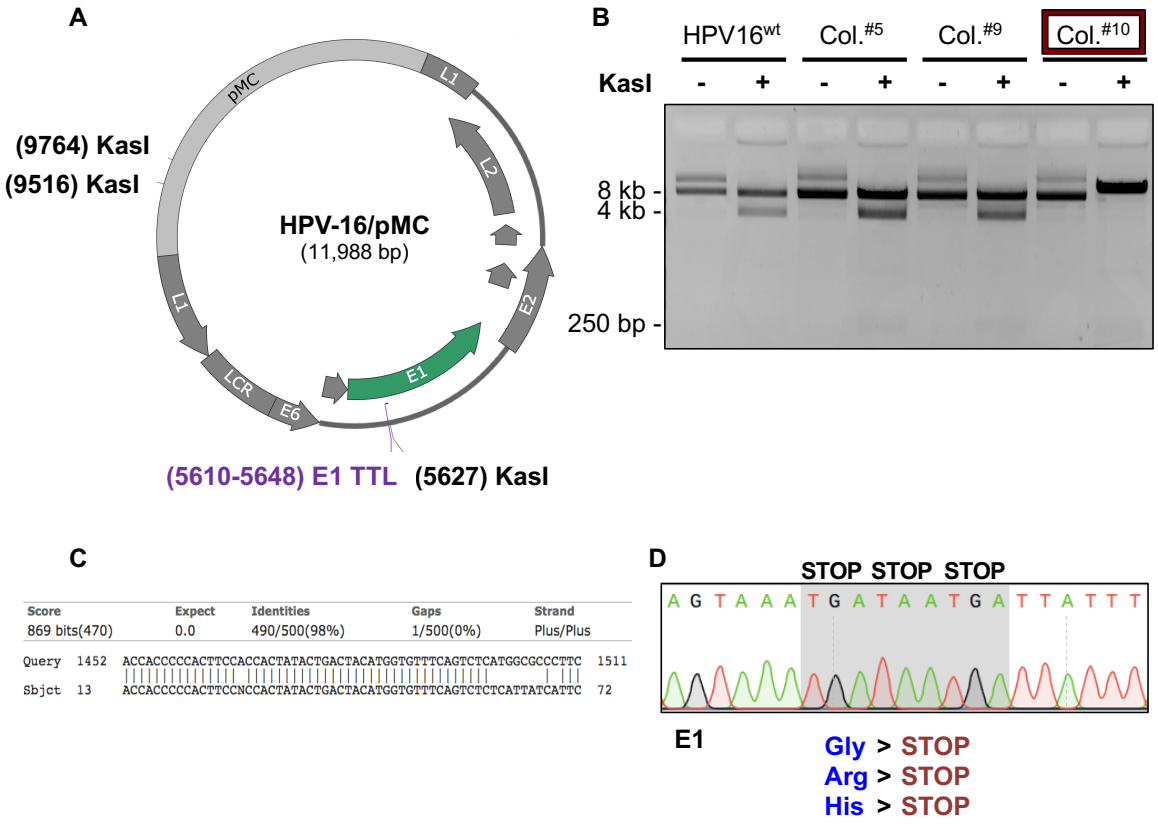

Figure A.1. Generating replication defective HPV-16 E1 mutant genomes. (**A**) Site-directed mutagenesis utilized primers to insert a translation termination linker (TTL) into the E1 open reading frame, which would remove a KasI restriction endonuclease site for enhanced colony screening. (**B**) Purified DNA was KasI endonuclease digested and run on 0.8% agarose gels. (**C**) NCBI sequence BLAST of wild-type HPV-16 and prospective HPV-16 E1 mutant genome from colony 10. (**D**) Sequencing results of DNA purified from colony 10 showed three sequential stop codons inserted into the E1 ORF.

Table A.1. E2 host interacting factors.

UniProt ID	Protein	Name	Reference
Q13227	AMF1	G-protein pathway suppressor 2	[207]
P10275	AR	androgen receptor	[208]
P38398	BRCA1	breast cancer type 1 susceptibility protein	[209]
O60885	BRD4	Bromodomain-containing protein 4	[170, 210-212]
Q07021	C1QBP	complement component 1 Q subcomponent-binding protein	[213]
Q14790	CASP8	Caspase-8	[214]
Q92793	CBP	CREB-binding protein	[215]
Q12834	CDC20	cell division cycle protein 20	[216]
Q9UM11	CDH1	Fizzy-related protein homolog	[216]
P49715	CEBPA	CCAAT/enhancer-binding protein alpha	[217]
P17676	CEBPB	CCAAT/enhancer-binding protein beta	[217]
O15519	cFLIP	FADD-like apoptosis regulator	[218]
Q8TD26	CHD6	chromodomain-helicase-DNA-binding protein 6	[219]
Q96FC9	CHLR1	DEAD/H box polypeptide 11	[85]
P09496	CLTA	clathrin light chain A	[220]
O95639	CPSF4	cleavage and polyadenylation specificity factor subunit 4	[221]
Q13618	CUL3	Cullin-3	[222]
Q09472	p300	histone acetyltransferase p300	[223]
Q96L91	P400	E1A binding protein p400	[212]

Table A.1. E2 host interacting factors (cont.).

UniProt ID	Protein	Name	Reference
P21802	FGFR2	Fibroblast growth factor receptor 2	[224]
P22607	FGFR3	Fibroblast growth factor receptor 3	[225]
Q9Y3R0	GRIP1	Glutamate receptor-interacting protein 1	[226]
Q00403	GTF2B	transcription initiation factor IIB	[220]
Q13547	HDAC1	histone deacetylase 1	[227]
Q92769	HDAC2	histone deacetylase 2	[227]
O15379	HDAC3	histone deacetylase 3	[113, 227]
O95235	KIF20A	Kinesin-like protein KIF20A	[220]
O00505	KPNA3	Importin subunit alpha-3	[228]
O15131	KPNA5	Importin subunit alpha-6	[228]
Q00987	MDM2	E3 ubiquitin-protein ligase Mdm2	[229]
P55209	NAP1	Nucleosome assembly protein 1-like 1	[230]
O75376	NCoR	Nuclear receptor corepressor 1	[113]
P48552	NRIP	Nuclear receptor-interacting protein 1	[231]
Q13416	ORC2	Origin recognition complex subunit 2	[175]
P09874	PARP1	Poly[ADP-ribose] polymerase 1	[232]
Q92831	PCAF	Histone acetyltransferase KAT2B	[233]
Q9UM63	PLAL1	Zinc finger protein PLAGL1	[226]
P53350	PLK1	Serine-threonine-protein kinase PLK1	[234]

Table A.1. E2 host interacting factors (cont.).

UniProt ID	Protein	Name	Reference
P27694	RPA1	Replication protein A	[235]
Q15047	SETDB1	Histone-lysine N-methyltransferase SETDB1	[227]
Q07955	SRSF1	Serine/arginine-rich splicing factor 1	[213, 236]
Q01130	SRSF4	Serine/arginine-rich splicing factor 2	[236]
Q16629	SRSF5	Serine/arginine-rich splicing factor 5	[236]
Q13309	SKP2	S-phase kinase-associated protein 2	[237]
P51531	SMARCA2	Probable global transcription activator SNF2L2	[106]
P51532	SMARCA4	Transcription activator BRG1	[238]
Q12824	SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	[238]
Q8IY18	SMC5	Structural maintenance of chromosomes protein 5	[170]
Q96SB8	SMC6	Structural maintenance of chromosomes protein 6	[170]
Q16637	SMN1	Survival motor neuron protein	[239]
P08612	SNRNP70	U1 small nuclear ribonucleoprotein 70kDa	[213]
P08047	SP1	Transcription factor Sp1	[240]
P21675	TAF1	Transcription initiation factor TFIID subunit 1	[241, 242]
P49848	TAF6	Transcription initiation factor TFIID subunit 6	[241]
Q15545	TAF7	Transcription initiation factor TFIID subunit 7	[243]
Q86VP1	TAX1BP1	Tax1-binding protein 1	[244]
P20226	TBP	TATA-box-binding protein	[241, 243, 245]

Table A.1. E2 host interacting factors (cont.).

UniProt ID	Protein	Name	Reference
P04637	P53	Cellular tumor antigen p53	[246]
Q00403	TFIIB	Transcription initiation factor IIB	[247]
Q9Y5LO	TNPO3	Transportin-3	[213]
Q92547	TOPBP1	DNA-topoisomerase 2-binding protein 1	[248]
P11387	TopoI	DNA topoisomerase 1	[249]
P62995	TRA2B	Transformer-2 protein homolog beta	[213]
Q13263	TRIM28	Transcription intermediary factor 1-beta	[227]
P63279	UBC9	SUMO-conjugating enzyme	[250]
Q96JC1	VPS39	Vam6/Vp239-like protein	[220]

Table A.2. Cell lines.

Cell Line	Media	Notes	Source
C33A	DMEM/10% FBS		D. Lowy
CIN612-9E	E-MEDIA	co-culture w/J2	L. Laimins
HaCaT	DMEM/10% FBS		N. Fusenig
HaCaT/pcDNA3	DMEM/10% FBS	200 ug/mL G418	n/a
HaCaT/HPV-31 E2	DMEM/10% FBS	200 ug/mL G418	n/a
HEK293TT	DMEM/10% FBS		J. Schiller
HEK293TTF	DMEM/10% FBS		R. Roden
HeLa	DMEM/10% FBS		D. Lowy
HepG2	DMEM/10% FBS		H. Guo
Huh-7	DMEM/10% FBS		H. Guo
J2 3T3	DMEM/10% FBS	mito. C treat prior to co-culture	H. Green
N/TERT	KSFM	hTERT immortalized	I. Morgan
N/TERT/HPV-16 E2	KSFM	hTERT immortalized	I. Morgan
NIKS	F-MEDIA	co-culture w/J2	B. Allen-Hoffman
NIKS/HPV-31	F-MEDIA	co-culture w/J2; 250 ug/mL G418	M. DeSmet
SH-SY5Y	DMEM/10% FBS		ATCC

Table A.3. Plasmids.

Plasmid	VECTOR	Resistance (Bacterial/Human)	SOURCE
pcDNA3	pcDNA3	Amp/G418	Addgene
pmCherry	pmCherry	Amp/G418	C. Lorson
HPV-16 L1/L2 (codon-optimized)	p16sheLL	Amp/Blasticidin	J. Schiller
FLAG-SMC6 (NM_001142286)	pcDNA3	Amp/G418	GenScript
HPV-31 HA-E1 (codon-optimized)	pcDNA3	Amp/G418	A. McBride
HPV-31 FLAG-E2 (codon-optimized)	pcDNA3	Amp/G418	A. McBride
HPV-31 FLAG-E2^{mt} (codon-optimized; aa207-372)	pcDNA3	Amp/G418	[174]
HPV-31 V5-E2 (codon-optimized)	pCIneo-V5	Kan/G418	T. Gilson
HA-εCOP	pcDNA3	Amp/G418	T. Gilson
MISSION shGFP (SHC002)	pLKO.1	Amp/G418	Sigma-Aldrich
MISSION shNSE3 (TRCN0000115778)	pLKO.1	Amp/G418	Sigma-Aldrich
MISSION shSMC6 (TRCN0000219949)	pLKO.1	Amp/G418	Sigma-Aldrich
cccHBV(+)/pSP	pSP	Amp	H. Guo

Table A.4. siRNAs.

siRNA	Target	Source	Notes
MISSION esiGFP	eGFP	Sigma-Aldrich (EHUEGFP)	heterogeneous siRNA mix targeting EGFP mRNA
MISSION esiSMC6	SMC6 (NM_024624)	Sigma-Aldrich (EHU026931)	heterogeneous siRNA mix targeting SMC6 mRNA
siScramble	Non-targeting	IDT (51-01-19-08)	scramble
siNSE3	NSE3	IDT (hs.Ri.NDNL2.13.1)	exon 1(NM_138704)
siSMC6	SMC6	IDT (hs.Ri.SMC6.13.2)	exon 21(NM_024624) exon 22(NM_001142286)

Table A.5. Antibodies.

Antibody	Target	Source/Catalog #	NOTES
Rabbit α-goat IgG[2] (control antibody)	heavy & light chain	Jackson (305-005-003)	1μL(ChIP)
mouse DM1a α-α-tubulin[1] (1º antibody)	α-tubulin	Sigma-Aldrich (T6199)	1:5000(WB)
Mouse M2 α-FLAG[1] (1º antibody)	FLAG	Sigma-Aldrich (F3165)	1:3000(WB) 2μL(co-IP)
Mouse 12CA5A1 α-HA[1] (1º antibody)	HA	Sigma-Aldrich (11583816001)	1:3000(WB) 2μL(co-IP)
Mouse α-SMC6[1] (1º antibody)	SMC6	Abgent (AT3956a)	1:1000(WB)
Mouse α-V5[1] (1º antibody)	V5	Invitrogen (46-0705)	1:3000(WB)
Mouse α-β-actin[1] (1º antibody)	β-actin	Sigma-Aldrich (A2228)	1:5000(WB)
Rabbit α-HPV-31 E2[2] (1º antibody)	HPV-31 E2	[175] (PAC 10008; final)	10μL(ChIP)
Rabbit α-NDNL2[2] (1º antibody)	NSE3	Abcam (ab198686)	1:1500(WB)
Rabbit α-SMC6[2] (1º antibody)	SMC6	Bethyl (A300-237A-M)	1:1000(WB) 2μL(ChIP; co-IP)
Rabbit α-V5[2] (1º antibody)	V5	Cell Signal Technologies (D3H8Q)	1:3000(WB) 2μL(co-IP)
Rat α-HPV-31 E1[1] (1º antibody)	HPV-31 E1	[175] (H14-4E_32731)	1:3000(WB) 2μL(co-IP)
mouse α-rabbit IgG (2º antibody)	light chain	Jackson (211-032-171)	1:10000(WB)
donkey α-mouse IgG (2º antibody)	heavy & light chain	Jackson (715-035-151)	1:10000(WB)
goat α-mouse IgG (2º antibody)	light chain	Jackson (115-035-174)	1:10000(WB)

[1]: monoclonal
[2]: polyclonal

Table A.6. Primers.

PRIMER SET	SEQUENCE (5'>3')	REFERENCE
HPV-31 L1	F: cacctccctcaggttctttg R: atggatcttccttgggcttt	[175]
HPV-31 E2BS (5')	F: cctgctcctcccaatagtca R: ggaccgggtgtacaactttt	[175]
HPV-31 E2BS (3')	F: ttgtgcaaacctacagacgcca R: agcttagttcatgcaatttccgagg	n/a
GM-CSF	F: acccagtccacctcactaat R: ttggtttcctttcagagcct	[251]
HPV-31 E1	F: agccacccaaattacgtagcac R: tcgctaatgtttgacattcctgttc	n/a
HPV-31 E1^E4	F: tgttaatgggctcatttggaa R: ggttttggaattcgatgtgg	[176]
HPV-31 E2	F: agcgttgtcagtatcaaaggc R: gctgcattgtccagtcctcat	n/a
HPV-31 E8^E2	F: gtggaaacgcagcagatggta R: ttcgatgtggtggtgttgttg	n/a
SMC6	F: gttggcgaaatgaaccggag R: cttgtcttggccttttggca	n/a
NSE3	F: ctcatcaacaccctggagcc R: atcatcaggaggcccgtagt	n/a
β-actin	F: gaggcactcttccagccttc R: cggatgtccacgtcacactt	[175]
GAPDH	F: cgcaggccggatgtgttc R: acgaccaaatccgttgactcc	n/a
cccHBV	F: gtctgtgccttctcatctgc R: acaagagatgattaggcagagg	n/a

Table A.7. SMI media. Media used for standard cell culture and media found most effective for mediating high infection efficiency using SMI.

CELL LINE	STANDARD MEDIA	SMI MEDIA
CaSki	DMEM/10% FBS	DMEM/10% FBS
CIN612-9E	E	E
HaCaT	DMEM/10% FBS	F
HaCaT	DMEM/10% FBS	F
HEK293TT	DMEM/10% FBS	DMEM/10% FBS
HEK293TTF	DMEM/10% FBS	DMEM/10% FBS
HeLa	DMEM/10% FBS	F
HepG2	DMEM/10% FBS	E
HFK	KSFM	F
Huh-7	DMEM/10% FBS	E
J2	DMEM/10% FBS	F
N2A	DMEM/10% FBS	F
NIKS	F	F
N/TERT	KSFM	F
SH-SY5Y	DMEM/10% FBS	DMEM/10% FBS
SiHa	DMEM/10% FBS	E

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CURRICULUM VITAE

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EDUCATION

Ph.D. **Indiana University**, Indianapolis, IN 46202 (2015 - 2020)

Microbiology & Immunology

Cancer Biology minor

M.A. **Ball State University**, Muncie, IN 47304 (2014 - 2015)

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Dean's Citation for Academic Excellence

B.S. **Ball State University**, Muncie, IN 47304 (2012 - 2014)

Biology

Biotechnology Certificate

Cum Laude

RESEARCH EXPERIENCE

- Characterizing the role of the SMC5/6 complex as a host restriction factor of high-risk human papillomavirus — 2016-20
  Primary Investigator: Dr. Elliot Androphy

- Optimization of human papillomavirus-based pseudovirus techniques for efficient gene transfer — 2018-20
  Primary Investigator: Dr. Elliot Androphy

- *Plasmodium falciparum* transmission in the development of malaria in highland versus lowland populations in Kenya — 2015
  Primary Investigator: Dr. Chandy John

- Proviral activation of latently HIV-1 infected monocytes — 2015
  Primary Investigator: Dr. Quigui Yu

- The role of TAL1 in T-cell acute lymphoblastic leukemia — 2013-2015
  Primary Investigator: Dr. James Olesen

- Quality control mechanisms of the ER translocon — 2014
  Primary Investigator: Dr. Eric Rubenstein

PUBLICATIONS

Ryan T. **Gibson** and Elliot J. Androphy. The SMC5/6 complex represses the replicative program of high-risk human papillomavirus type 31. *Pathogens*, 2020. **9**(10).

Gilson, T.D.*, **Gibson**, **R**.**T**.*, and Androphy, E.J. Optimization of human papillomavirus-based pseudovirus techniques for efficient gene transfer. *Sci Rep*, 2020. **10**(1): p. 15517. ***[*co-first author]***

Crowder, J.J., Geigges, M., **Gibson, R.T.**, Fults, E.S., Buchanan, B.W., Sachs, N., Schink, A., Kreft, S.G., and Rubenstein, E.M. Rkr1/Ltn1 Ubiquitin Ligase-mediated Degradation of Translationally Stalled Endoplasmic Reticulum Proteins. *J Biol Chem*, 2015. **290**(30): p. 18454.

Ryan T. **Gibson** and James B. Olesen. Development of an immunocytochemistry protocol for the study of TAL1 mediated apoptotic resistance in Jurkat cells. Muncie, Indiana: Ball State University. 2015. Print.

PRESENTATIONS

Gibson, R.T. Elucidating the role of SMC5/6 in the life cycle of high-risk human papillomavirus. 2018-19. Indiana University School of Medicine.

Gibson, R.T., Patel, J., Victorino, J. 2017. Think, Talk, and Study Like a Graduate Student. Indiana University School of Medicine.

Gibson, R.T., Varberg, K., Victorino, J. 2016. Think, Talk, and Study Like a Graduate Student. Indiana University School of Medicine.

Gibson, R.T. 2015. Suitability of αSNAP for inhibition of Staphylococcus aureus infection. Ball State University.

HONORS AND AWARDS

- ASPiRE Grant Recipient 2015
- Ball State University Dean's Citation for Academic Excellence 2015
- Ball State University Certificate of Achievement 2015
- Ball State University Dean's List 2012 – 2014
- Phi Theta Kappa Honor Society Presidential Scholarship 2012

PROFESSIONAL AFFILIATIONS

- Sigma Xi International Research Society Member 2014 – Present
- Omicron Delta Kappa Honor Society Member 2014 – Present
- Golden Key Honor Society Member 2014 – Present